1927

A study of some of the lactobacilli

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A STUDY OF SOME OF THE LACTOBACILLI.

BY

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for the Degree of

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Approved

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GENERAL INTRODUCTION.

Certain members of the genus Lactobacillus (Beijerinck) are of particular importance to the dairy industry; namely, L.acidophilus (Moro), L.bulgaricus (Grigoroff), and L.casei (v.Freudenreich). Of these, the first two have in recent years occasioned an increasing amount of interest because of their suggested therapeutic value in the treatment of intestinal disorders and because they are so alike culturally that some doubt still exists as to definite differentiating characters. The third, although recognized several years prior to the others, has received comparatively little attention except in its relationship to the ripening of certain types of cheese.

The purpose of this study has been to investigate various organisms usually considered as belonging to these three species with a view to correlating some of the more outstanding characters; such a correlation of characters was considered of value in proper identification and classification.

The work has been organized and presented in four parts. Part I is a discussion of the distribution and

isolation of the organisms studied. Part II includes studies on growth temperatures, on the effects of surface tension of the medium on growth, on the character and amount of acid produced in milk, and on the proteolytic action of the organisms in milk. Part III includes observations on the physiological adaptability of some of the organisms as determined by experiments with humans and rats. Part IV includes a comparison of sterilization and pasteurization of milk for the preparation of acidophilus milk, and a study of the effect of different temperatures on the viability of L.acidophilus.

In each part a review of the literature bearing on that phase of the subject has been given. No attempt has been made to make these complete inasmuch as excellent reviews have appeared at intervals in the natural development of the various problems.

PART I.

DISTRIBUTION AND ISOLATION.

Introduction.

Microorganisms are influenced by their environmental conditions and adapt themselves in various ways, often to such an extent that they cannot exist where the conditions are not similar to those to which they have become accustomed. This adaptation of organisms to a favorable environment

may be very strict in its limitations as is the case with strictly parasitic types which may require very definite host conditions, or it may be more general as with members of the colon-aerogenes group which adapt themselves to wide variations in environment. When considering certain groups of organisms the source from which they are isolated constitutes a point of considerable importance; when correlated with other definite characters, this often aids in the proper identification and classification of species in the group. Consideration of the sources of the organisms studied in this work then should be of considerable value when correlated with various characters to be discussed in part II.

Review of Literature.

In extended studies on the ripening of Swiss cheese von Freudenreich (1891) first named as the causal agent Bacillus alpha, but a few years later (1899) he ascribed the leading role to Bacillus epsilon. Some years later he and Thöni (1904) described the group of organisms more completely.

Orla-Jensen (1906) confirmed the statements of von Freudenreich that B.casei E was the most important agency in the ripening of Emmental cheese and that it reached its maximum numbers soon after the cheese had been made.

Up to this time B.casei E had apparently been considered only in connection with Swiss cheese, but Fascetti (1907) found that a starter of Bacillus casei E and Mycoderma thöni used in making Grana cheese gave the characteristic structure and savor of Gruyere cheese within a three months period.

In the principal work on the bacteriology of cheddar cheese in this country Evans, Hastings, and Hart (1914), Eldredge and Rogers (1914), Hart, Hastings, Flint and Evans (1914), and Hucker (1922) have all stressed the extensive presence of the lactobacilli of the Bacterium casei and B.bulgaricus group and their role in the ripening of this type of cheese.

Samarani (1907) reported that the Sardinian fermented milk, Cieddu, contained only two forms of microorganisms, one of which was a variety of Bacterium lactis acidi and the other a variety of Bacillus casei of Freudenreich.

Hastings and Hammer (1909) during an investigation of the cause of high acidities in milk, isolated the causal organism, which they stated was a bacillus closely related to B.bulgaricus and to B.casei E. They found the bacillus widely distributed in milk, butter, and cheese.

Burri and Staub (1915 and 1917) failed to cor-

roborate the work of von Freudenreich and Orla-Jensen as to the role of B.casei E in the ripening of Swiss cheese. They found that this organism was absent after the fifth month and present only in small numbers at the end of the third month. Cheese containing large numbers of B.casei E in the earlier stages were soon overcrowded with strains of B.casei(alpha) and B.casei(gamma). They reasoned that since B.casei E was not present in fully ripened cheese it was not the chief cause of the ripening.

Recently Sherman and Stark (1927) reported the predominance of L.casei as compared to L.bulgaricus and L.acidophilus in both Grade A and ordinary milk. Of the Grade A samples, 71% contained L.casei in numbers of at least one per cc., while only 12% contained L.bulgaricus or L.acidophilus in like numbers. In the ordinary milk L.casei was present in 94% of the samples in excess of ten per cc., while only 2.3% contained the other lactobacilli in like numbers.

In the field of intestinal bacteriology the work of Escherich (1886) stands out as the first systematic study of intestinal types. It remained for Moro (1900) and Tissier (1900) to isolate two types which Escherich had observed but failed to isolate. The former inoculated fecal material from infants into beer wort bouillon for enrichment and then by plating the sediment on acid beer wort agar obtained nearly pure seedings of the species

which he named Bacillus acidophilus. He claimed this species as the predominating type in the stool of the normal nursling infant. Tissier called his type Bacillus bifidus and made the same claims for it which Moro had made for Bacillus acidophilus. He was substantiated in this by several workers and his contention was finally also admitted by Moro (1905).

The same year that Moro announced his new species, Finkelstein (1900), working independently, reported the isolation of an organism which he considered identical with that of Moro. He reported the method which he used for isolation as one which Heymann had been using for two years, and it has been used with modifications almost exclusively since then by the many workers in this field. It consists of inoculating fecal material into 0.5-1% acetic acid bouillon containing 2% dextrose, incubating for 24 to 48 hours and then plating on glucose agar.

For isolation purposes Kendall (1910b) used N/20 acetic acid bouillon, transferring 1/2 cc. at 48 hour intervals three times, and then plating. He proposed the term "aciduric" rather than "acidophilic" for the group; Rodella (1901) several years before had objected to the term "säureliebenden." Kendall presented an excellent review of the literature up to this time.

The number of articles published show that about this period interest in this group of intestinal organisms received an impetus which has continued to the present. Cammidge (1914) mentions acid dextrose broth as "specific" for the isolation of B.acidophilus and B.bifidus. Torrey (1915) found in the stools of typhoid fever patients studied that B.acidophilus was the only organism aside from yeasts capable of growing in acetic acid glucose broth. He used infusion bouillon containing 1% dextrose adjusted to N/5, N/10 and N/20 acetic acid inoculated with 0.5 cc. of a fecal suspension of 500 mg. in 50 cc. of normal salt solution. Later Torrey (1917) reported the use of beef liver glucose agar "adjusted to +4 acid" in the examination of feces as a means of inhibiting the growth of streptococci and most strains of B.coli and for differentiating between B.acidophilus and B.bifidus.

Cannon (1924) isolated 64 strains of organisms from the feces and sputum of adults by means of 0.25% acetic acid, unneutralized dextrose infusion broth with a pH of 4.8 - 5.0. He made transfers three times at 24 hour intervals before plating on whey or dextrose yeast agar. In the same year Goldman (1924) mentioned N/10 acetic acid broth as of help in isolating B.acidophilus from feces of humans.

Kulp and Rettger (1924) obtained their cultures from the feces of rats either by direct plating after liberal feeding with dextrin and lactose or by using Heymann's glucose acetic acid broth as an enrichment medium without preliminary carbohydrate feeding.

In isolating cultures from the feces of calves, Orcutt (1926) made use of standard agar, plus horse blood, adjusted to pH 6.8 - 5.0; in this medium B.acidophilus colonies formed small greenish zones.

A method not involving the use of enrichment media was suggested by Briedigam and Chang (1924) in which colonies could be picked from a heavily seeded plate by means of a capillary pipette manipulated by a microscope, the colony fished being in focus under another.

Sources other than the gastro-intestinal tract of man and various animals have been reported by several investigators. Heinemann and Hefferan (1909) offered the suggestion that B.bulgaricus through its lactic acid producing properties might be the cause of caries of the teeth, while Howe and Hatch (1917) isolated organisms which they found to be the constant and predominant flora of dental caries; these they considered identical with the Moro-Tissier group.

McIntosh, James and Lazarus-Barlow (1922) and

Rodriguez (1923) confirmed this work and considered L.acidophilus the etiological factor. McIntosh and his co-workers suggested the name L.acidophilus-odontolyticus for the group. Sierakowski and Zajdel (1924) confirmed finding the types reported by these investigators. At about the same time Clarke (1924) reported finding B.acidophilus only in advanced cases of caries and named S.mutans as the causal agent. In this country Bunting and Palmerlee (1925) found B.acidophilus present in 100% of 73 cases of initial caries lesions; in 94% of advanced caries; and in 16% of mouths immune to caries. They could see no reason for a different name for the groups on the basis of morphology or fermentation tests.

In a study of the acid bacilli of the normal vaginal secretions, Jötten (1922) decided that Doederlein's bacillus and B.acidophilus were identical.

Grigoroff (1905) isolated a rod form from Bulgarian podvaka, the starter for "kisselo-mleko", by inoculating into sterile milk, then making several dilution tubes in lactose gelatin, and picking colonies from them. This rod, which is now called L.bulgaricus, he called Bacillus A.

Heinemann and Hefferan (1909) reported a study of Bacillus bulgaricus isolated from the feces of humans, cows, and horses, from cattle feed, soil, market milk,

human saliva, gastric juice, cornmeal, sauerkraut, olive juice, dill pickles and pepper mango. They inoculated the material into 0.5% acetic acid, 2% glucose broth, incubated 24 hours, then transferred to litmus milk from which they plated on agar to which 0.5% acetic acid was added.

Stevenson (1911) confirmed the findings of Heinemann and Hefferan, and of Hastings and Hammer as to the wide distribution of the lactobacilli, finding them in market milk, Swiss cheese, Deutscher Käse, Scotch cheddar cheese, sauerkraut, human saliva, feces of cows, and soil. For isolation purposes he used yeast whey for enrichment and whey agar with chalk as a plating medium.

In a study of the Boas-Oppler bacillus from three cases of carcinoma Galt and Iles (1915) concluded that the organism was identical with B.bulgaricus or showed only minor differences. They used whey as an enrichment medium before plating on "nasgar".

Hunter and Bushnell (1916) found the Bulgarian type to be the predominating organism in the fermentation of normal silage. Their results were confirmed by the work of Sherman (1916), and Heinemann and Hixson (1921).

Clark (1917), in discussing the acid production of B.bulgaricus in artificial media stated that he found the

same pH in silage juice and spontaneously soured corn meal gruel, in both of which others had found B.bulgaricus to be dominant.

Allen (1919) reported the isolation of B.bulgaricus from viscous starch and gluten liquors in the wet process of manufacture of products from corn and from the corn as it arrived in the cars.

Orla-Jensen (1921) stated that he had never succeeded in finding Thermobacterium bulgaricum in the feces of adults even after large daily doses of yoghurt, nor in those of an infant constantly fed on milk inoculated with a few drops of yoghurt, but by inoculating a little of the feces into milk kept at 45° C. the yoghurt rods were obtained as a pure culture.

As noted above Sherman and Stark (1927) have recently reported the presence of L.bulgaricus in milk, although in much lower numbers than L.casei.

Methods.

The cultures used in this study were obtained by original isolations from dairy products and the feces of humans and animals, and from research and commercial dairy laboratories.

The L.casei strains were isolated from cheddar cheese or milk by using milk as an enrichment medium and

then plating, except in one case in which the cheese was plated direct. When cheese was the source a plug was taken with a sterile trier after scraping off a small area of the surface with a sterile spoon. The lower end of the plug was then cut into a Petri dish by pressing the cheese on the edge of the dish. From these plugs pieces were removed with a heavy transfer needle, placed in small bottles of sterilized skimmed milk and incubated at 37° C. under aerobic conditions for one week. Plates were then poured with whey agar and from them colonies were picked into tubes of litmus milk. The presence of the Gram positive rods in the tubes was determined by the usual staining methods.

The strains isolated from milk were obtained by incubating fairly large samples of raw milk at 37° C. for 7 to 10 days, at which time microscopic examination showed the presence of rod forms. The usual method of plating and picking colonies mentioned above then yielded the cultures for study.

The fecal specimens were taken in sterile Petri dishes and brought to the laboratory immediately so that in only one instance was the material more than one or two hours old when the work of isolation was begun.

In the isolation of the cultures considered as

L.acidophilus two principal procedures were followed with success; a dilution method and the Heymann acetic acid bouillon method. In the former the fecal material was inoculated by means of a loop into a sterile water blank and from this into a series of twenty or more tubes of litmus milk, using a varying amount of the dilution. At the same time that these litmus milk inoculations were made, plates were poured with whey agar for direct examination. Transfers into milk were made from the tubes of the highest dilution which showed evidences of growth at 37° C. within two to three days. After several such transfers for enrichment purposes the milk was plated on whey agar and colonies picked for purification.

In the Heymann acetic acid method the feces were inoculated heavily into beef infusion bouillon containing 2% dextrose and adjusted to N/10 acetic acid; the tubes were held at 37° C. At intervals of 24 hours, two successive transfers to other tubes of the acid broth were made, using pipettes in order to secure heavy inoculations. Twenty-four hours after the last transfer, plates were poured from the original tube and the two transfers. Colonies developing on these plates were examined under the microscope and the various types picked into litmus milk. By subsequent examination of the tubes of milk showing

growth, the cultures for study were selected.

Regular gradations in the size of the cells existed which would have made it possible to divide the cultures into several groups although there would have been no sharp line of demarcation between the various groups. Variations in size were also apparent in the cells of each culture, even after purification by repeated platings. This pleomorphic character among cultures of these organisms, which has been constantly noted by various investigators, is one which is particularly confusing when examining such stained material as a fecal smear where all gradations in the size of the cells are seen, and also in the examination of a culture when doubt exists as to its purity. Such wide variation in size in the supposedly pure cultures would have complicated the division into several groups, as one culture might, from the appearance of individual cells, have been placed in more than one group. It was decided that for purposes of this discussion two general groups would be sufficient and the least confusing, so the cultures were designated as large or small. With these two groups there was no doubt as to the proper placing of the cultures since the large organisms were definitely rather thick rods varying from short to very long forms, and the small types were all

slender rods, with a similar variation in length.

Results Obtained.

SOURCES:

The data showing the sources of the 86 cultures studied are presented in Table I; a statement of the relative size of each culture is included.

Nine cultures were obtained from research and commercial laboratories as representative L.acidophilus strains; six of these were classed as large and three as small. Seven representative L.bulgaricus cultures, including one of the original Metchnikoff strains, were secured from research laboratories; of these, five were classed as large and two as small.

Sixteen cultures considered as L.acidophilus were isolated from the fecal matter of man and animals; seven were classed as large and nine as small. Five of the seven large strains were from the feces of young calves and two from the feces of rats. Of the nine small strains seven were secured from the feces of infants from three weeks to one year of age, one from an adult, and one from a rat.

Fifty-four cultures considered as L.casei were secured by isolation and from a research laboratory. Of these 37 were isolated from milk and well cured cheddar cheese, and 17 were secured from the stock culture collec-

TABLE I

Sources and relative sizes of organisms studied.

Culture number	Source	Rel. size	Culture number	Source	Rel. size
A1 *	Commercial lab.	small	C12	Cheddar cheese	small
A3	" "	large	C13	Raw milk	large
A4	" "	"	C14	" "	"
A5	Research lab.	"	C16	" "	"
A6	Commercial lab.	"	C17	" "	small
A7	Research lab.	"	C18	" "	large
A8	" "	"	C19	Cheddar cheese	small
A9	Commercial lab.	small	C20	" "	"
A10	" "	"	C20a	" "	large
B1	Research lab.	"	C21	" "	small
B2	" "	large	C22	" "	"
B3	" "	"	C22a	" "	"
B5	" "	"	C23	" "	"
B6	" "	"	C24	Raw milk	large
B7	" "	small	C25	" "	small
B8	" "	large	C26	" "	large
S1	Infant feces	small	C27	" "	small
S5	" "	"	C28	" "	"
S6	" "	"	C29	" "	"
S7	Adult "	"	C29a	" "	large
S8a	Calf "	large	C30	" "	"
S8b	" "	"	C31	" "	"
S8c	" "	"	C32	" "	small
S9a	" "	"	C32a	" "	large
S9b	" "	"	C33	" "	"
S10	Rat "	"	C34	" "	"
S11	" "	"	C36	" "	small
S12	Infant "	small	C40	Research lab.	"
S15	" "	"	C41	" "	"
S15a	" "	"	C42	" "	"
S16	" "	"	C43	" "	"

S6	"	"	"
S7	Adult	"	"
S8a	Calf	"	large
S8b	"	"	"
S8c	"	"	"
S9a	"	"	"
S9b	"	"	"
S10	Rat	"	"
S11	"	"	"
S12	Infant	"	small
S15	"	"	"
S15a	"	"	"
S16	"	"	"
S17	Rat	"	"
C1	Research lab.		"
C2	Cheddar cheese		"
C3	Raw milk		"
C3a	" "		"
C4	" "		"
C5	Cheddar cheese		"
C6	"	"	"
C7	"	"	"
C8	"	"	"
C9	"	"	"
C10	"	"	"
C29	"	"	"
C29a	"	"	large
C30	"	"	"
C31	"	"	"
C32	"	"	small
C32a	"	"	large
C33	"	"	"
C34	"	"	"
C36	"	"	small
C40	Research lab.		"
C41	"	"	"
C42	"	"	"
C43	"	"	"
C44	"	"	"
C45	"	"	"
C46	"	"	"
C47	"	"	"
C48	"	"	"
C49	"	"	"
C50	"	"	"
C51	"	"	"
C52	"	"	"
C53	"	"	"
C54	"	"	"
C55	"	"	"

* A = *L. acidophilus* cultures from laboratories

B = *L. bulgaricus* cultures from laboratories

S = *L. acidophilus* cultures isolated

C = *L. casei* cultures

tion maintained by the Dairy Bacteriology Laboratory of the Iowa Agricultural Experiment Station. Sixteen of the latter were isolated about twelve years previous to this study from samples of milk including one from Canada, one from Denmark, one from the Isle of Wight, and three from Ireland, while the source of the other was not known. Of the 37 cultures isolated, 15 were obtained from cheddar cheese and 22 from raw milk. Of the 15 cultures from cheese, one was classed as large and 14 as small, while of the 22 from milk, 12 were large and 10 small. Among the 54 L.casei cultures there were 13 classed as large and 41 as small.

Of the 86 cultures included in the study, 31 were classed as large and 55 as small.

Several writers have suggested the possibility of morphological changes in organisms carried through a long series of transfers, but no such changes were seen during the course of this investigation. So far as could be determined, the size of the cells in every culture was the same at the conclusion of the work as at the beginning.

METHODS OF ISOLATION:

FROM DAIRY PRODUCTS: The use of milk for enrichment resulted in the isolation of fourteen cultures from

cheddar cheese; failure resulted in only one instance. One culture was obtained by the direct plating of the cheese; this was the only instance in which cheese was plated direct since previous experience had shown the enrichment method to be more certain.

The isolations from milk presented no particular difficulties except that in several instances heavy mold growth interfered with picking colonies so that several platings were necessary for purification. Twenty-two cultures were obtained by this method. Failures resulted in three cases in which the milk was of poor quality and was rapidly peptonized.

FROM FECAL MATERIAL: Examinations were regularly made of Gram stained smears of all fecal specimens and tubes of litmus milk were inoculated directly from the specimens and the character of the resulting fermentation observed. When large numbers of cocci or Gram-negative organisms were present, the curd developing was invariably of the stormy type with a considerable amount of whey. Usually when considerable numbers of small Gram-positive rods were present the curd formed was fairly smooth and with only a slight amount of whey; in one case, however, although the smear seemed to contain almost exclusively small Gram-positive rods the fermentation was

quite stormy and stained smears of the milk showed the presence of large numbers of cocci and irregular staining rods which failed to appear on whey agar plates or in successive transfers in milk. Several attempts at isolation in this case were unsuccessful, the probable explanation being that the organisms were the bifid rods described by Tissier which require anaerobic conditions.

The dilution method was successfully employed in three cases. From the series of litmus milk dilutions, tubes were found in which the curd was smooth and firm, with only a slight amount of whey; the litmus was first reddened and then reduced, leaving the characteristic red band at the top. The desired organisms were then readily isolated by plating and picking colonies into litmus milk. In two other instances in which dilution tubes were prepared, although coagulation occurred in some, no Gram-positive rods were found in smears and no further attempt was made to isolate organisms from them.

The Heymann acetic acid bouillon method was successfully employed with six fecal specimens from which ten cultures were isolated. The best plates from the standpoint of the distribution of desired colonies were obtained from the original heavily inoculated acid bouillon tube and the first transfer. Another point of interest

in this connection is that one specimen was inadvertently held in the refrigerator several days before work was begun on it and yet the isolation was successful. Possibly the necessity for haste in working with fecal specimens has been overstressed, as Jordan (1926) has indicated in work with stored stools of typhoid patients. The acid bouillon method was also used in two cases which resulted in complete failures to isolate the desired types, and in a third which was discontinued when one of the other methods resulted in an isolation. One of the failures was in the case previously mentioned in which the fecal smears apparently consisted almost exclusively of Gram-positive rods. The other failure may very likely have been due to the fact that at that time a much lower acidity than N/10 was used and the other types present were able to outgrow the Gram-positive rods which were present in much smaller numbers according to the appearance of the fecal smear. It was also noted that this particular specimen was much darker than others and had the most pronounced disagreeable odor.

Plates poured directly from fecal suspensions in three cases contained a sufficient seeding of the proper colonies to assure isolation of the rods by the first picking. In two of these instances the subjects from whom

the fecal material was secured had been consuming milk fermented with culture A1 for some time previous to supplying the specimens, while in the third the rat subject had been on a carbohydrate diet for some time. In later work numerous cultures not included in the main study were isolated in this manner during routine examination of feces from humans and rats. Milk inoculated directly with the feces in such cases was practically always coagulated smoothly with very little or no gas or whey.

Discussion of Results.

Wide morphological variations were found within the pure cultures and in the various cultures, whether from the same or different sources. All of the L.acidophilus cultures isolated from human fecal material and most of those from rat feces were small, while those from calf feces were large. Similar variations were evident among the L.casei cultures; all of the strains isolated from cheddar cheese except one were small, while both large and small types were readily isolated from milk. This suggests the possibility that environmental conditions in cheese are not favorable for the development of the large types although they were very probably present in the milk used in making the cheese.

Such variations in size indicate that size alone

can not be depended upon for differentiation when considering organisms belonging to these species. It is probable, however, that this character is valuable when correlated with other characters.

Consistent results secured in isolating cultures of L.casei from milk and cheddar cheese confirm numerous reports on the prevalence of these lactobacilli in milk and cheese. Success in isolating organisms of the L.acidophilus type from the fecal material of human infants, an adult, calves and rats is in agreement with the reports of various investigators. The fairly regular appearance of the slender Gram-positive rods in the feces of infants still on a milk diet also substantiates other investigations. This further suggests the possible value of a milk diet in establishing these types in the intestinal tract.

In the isolation of L.casei the use of milk for enrichment purposes proved very satisfactory. Results obtained with various methods of isolating L.acidophilus seemed to indicate the advisability of using the Heymann acetic acid medium for enrichment before plating out. The dilution method and direct plating of fecal material were also successful but except under favorable conditions the more certain method seemed to be the use of an acid

medium. These results agree with those of various investigators most of whom have depended entirely upon acid media to favor the development of these lactobacilli.

PART II.

IMPORTANT CHARACTERS.

Introduction.

Various characters have been suggested at different times for differentiating between L.bulgaricus and L.acidophilus. Among the earliest of these was the fermentation of carbohydrates, particularly maltose, which L.bulgaricus was presumably unable to ferment. However, Grigoroff (1905) in his original description of this organism named maltose as one of the sugars which it was able to attack. A difference in the ability to ferment maltose was made practically the entire basis for differentiation between L.bulgaricus and L.acidophilus by Rahe (1914 and 1915), and was the deciding factor used by Rettger and Cheplin (1921) when they were otherwise in doubt as to the identity of organisms forming a colony typical of both species. Many investigators have reported inconsistent results on carbohydrate fermentations and this inconsistency has even led to the proposal of new species and the division of existing species into several groups on this basis. More recently it has become quite generally

accepted that this is not a satisfactory criterion upon which to base the differentiation of L.bulgaricus from L.acidophilus, though it may be of value when correlated with other characters.

Colony formation has proven unreliable because of the close similarity between the characters of the colonies formed by the various types, and because the appearance of the colonies is greatly influenced by such factors as the character of the medium in which they are formed and the crowding of colonies on the plates. There appears to be no definite correlation between the morphology of the organism and the type of colony formed.

At one time the total amount of acid produced by the various species was considered sufficiently distinct to serve as a means of differentiation, but such wide variations have been reported that it appears advisable not to lay too great stress upon this particular character alone. The type of acid formed is also a point upon which there has been little agreement.

The temperature at which L.bulgaricus grows has been generally considered to be quite high within the optimum range for the lactobacilli; some reports have indicated that both L.casei and L.acidophilus are also able to grow at the high temperature commonly ascribed to

L.bulgaricus. Because of the almost thermophilic character of the latter species it has not been generally considered that it could grow at temperatures much below 30° C., while L.casei especially is regularly known to grow at the low temperatures used in curing the types of cheese in which it occurs. Probable variations in the ability of these three species to grow at 45° C. and 15-20° C. were suggested by Sherman (1921); this was restated with modifications by Sherman and Stark (1927) in more definite form and suggested as a means of differentiation.

Variations in the ability of L.bulgaricus and L.acidophilus to grow in media with a reduced surface tension were first reported by Albus and Holm (1925). Their results were evidently quite definite and suggested a method which might prove of considerable value in differentiating between these two species.

Although other methods have been suggested for separating these species, such as their serological relationships and their staining reactions, the present study has been confined to what appeared to be the more important characters of the organisms with a view to determining whether or not any correlation existed which would be of value in differentiating between the various

types. The L.casei group has been reported in only a few instances in connection with studies of L.acidophilus and L.bulgaricus so that some information on their interrelationship was particularly desirable.

The results of the studies considered in part II are arranged under four headings: growth temperatures, effect of surface tension on growth, character and amount of acid produced, and protein decomposition.

GROWTH TEMPERATURES

The optimum temperature range of most of the lactobacilli lies between 30° C. and 42° C. but within more narrow limits the optimum range for the three species under discussion may be given as 37° C. to 42° C. Ability to grow at temperatures above or below the optimum, if sufficiently definite, might well serve as a differentiating character. That L.bulgaricus is able to grow at a higher temperature than most of the lactobacilli has long been recognized, while the prevalence of the L.casei types in cheese cured at low temperatures might lead one to anticipate a difference in the ability of one to grow at temperatures favorable to the growth of the other.

Review of Literature.

Moro (1900) reported that B.acidophilus did not

grow at 20-22° C. and that its optimum temperature was 39° C.

Hastings and Hammer (1909) in their study of high acid producers from milk, cheese, and butter, noted growth at 50° C. and stated that the probable optimum was between 40° C. and 50° C. They found that growth at 20° C. in milk was slow but that eventually a high acidity was produced.

Heinemann and Hefferan (1909) stated that the Bulgarian bacillus did not grow at ordinary room temperature.

In discussing the use of milk fermented with L.bulgaricus for the preparation of lacto, Mortensen and Hammer (1913) stated that cultures could be propagated at room temperatures but that growth was quite slow; the usual higher temperatures were advocated.

It seems an anomoly that L.bulgaricus with the highest temperature requirements for growth among these lactobacilli should be found so widely distributed where the temperature would normally be much lower, as it is, for instance, in soil. In this connection Barthel (1919) pointed out that with B.casei epsilon(B.bulgaricus) better growth is obtained in acid soil (pH 5.0 - 6.0) at 22-24° C. than at 38° C. in spite of the almost thermophilic char-

acter of the organism in laboratory media.

In some notes on the lactobacilli, Sherman (1921) pointed out that L.bulgaricus did not grow at 15° C. and little or not at all below 20° C., while L.casei and the intestinal types grew at this temperature. Quite recently Sherman and Stark (1927) stated that L.bulgaricus and L.acidophilus grow at 45° C. while L.casei does not, and that at 15° C. L.casei will grow while the other two types will not, suggesting these facts as a possible means for differentiation.

Rettger and Cheplin (1921), in discussing the preparation of acidophilus milk, stated that slow coagulation occurred at 20° C. while below this temperature little or no change occurred in the appearance of the milk during the first three or four days.

Kulp and Rettger (1924) reported the growth of both L.acidophilus and L.bulgaricus at 20-25° C. as very slow, when evident at all. One bulgaricus culture curdled milk in 12 days, and two in 30 days. No other strain which they studied produced a curd and only a few caused reddening of the litmus.

Methods.

The most favorable growth temperature for routine work with the cultures was found to be 37° C., so they

were incubated at this temperature in litmus milk for 24 to 48 hours and then held at room temperature until the next transfer in ten days to two weeks. In studying the effect of temperature on growth the cultures were inoculated into litmus milk and held at room temperature, at 45° C., and at 15-20° C. for seven days. Changes in the appearance of the litmus as compared to check tubes were considered as evidences of growth. If any doubt existed as to a reddening of the litmus, smears were stained and examined for the presence of the organisms in numbers sufficient to indicate multiplication of those inoculated into the milk.

Results Obtained.

The data obtained on the growth of 88* cultures in milk during seven days at 45° C., room temperature (approximately 25° C.), or 15-20° C. are presented in Table II.

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* Cultures A3a and A3b which do not appear in Table I were obtained from culture A3 when checking that culture for purity during the work on the type of acid produced; they have been carried throughout the study, although they have not been discussed unless differences of significance occurred between them and culture A3.

TABLE II

Growth in milk at different temperatures during seven days.

Culture	45° C.	Room	15 - 20° C.	Culture	45° C.	Room	15 - 20° C.
A1	- *	+ +	+ +	C10	+ +	+ +	+
A3	+ +	-	-	C12	-	+ +	+
A3a	+ +	-	-	C13	-	-	-
A3b	+ +	-	-	C14	-	-	-
A4	+ +	-	-	C16	-	-	-
A5	+ +	-	-	C17	-	+ +	+
A6	+ +	+	-	C18	-	-	-
A7	+ +	-	-	C19	-	+ +	+ +
A8	+ +	+	-	C20	-	+ +	+
A9	+ +	+ +	+	C20a	+ +	+	-
A10	+ +	+ +	+	C21	-	+	+
B1	-	+ +	+	C22	+	+ +	+
B2	+ +	+	-	C22a	+	+ +	+
B3	+ +	+ +	-	C23	-	+ +	+
B5	+ +	+ +	-	C24	-	-	-
B6	+ +	+	-	C25	-	+ +	+
B7	+ +	+ +	+	C26	-	-	-
B8	+ +	+	-	C27	-	+ +	+
S1	-	+ +	+	C28	-	+ +	+
S5	-	+ +	+ +	C29	-	+ +	+
S6	-	+	+	C29a	-	-	-
S7	-	+ +	+	C30	-	-	-
S8a	+	-	-	C31	-	-	-
S8b	-	-	-	C32	-	+ +	+
S8c	-	-	-	C32a	-	-	-
S9a	+	-	-	C33	-	-	-
S9b	-	-	-	C34	-	-	-
S10	-	-	-	C36	-	+ +	+
S11		+	+	C40	+ +	+ +	+

S1	-	+ +	+	C28	-	+ +	+
S5	-	+ +	+ +	C29	-	+ +	+
S6	-	+	+	C29a	-	-	-
S7	-	+ +	+	C30	-	-	-
S8a	+	-	-	C31	-	-	-
S8b	-	-	-	C32	-	+ +	+
S8c	-	-	-	C32a	-	-	-
S9a	+	-	-	C33	-	-	-
S9b	-	-	-	C34	-	-	-
S10	-	-	-	C36	-	+ +	+
S11	-	+	+	C40	+ +	+ +	+
S12	-	+ +	+	C41	-	+ +	+
S15	-	+ +	+	C42	-	+ +	+
S15a	-	+ +	+	C43	+	+ +	+ +
S16	-	-	-	C44	-	+ +	+
S17	-	-	-	C45	-	+ +	+
C1	-	+ +	+ +	C46	+	+ +	+
C2	-	+ +	+	C47	-	+ +	+
C3	+ +	+	-	C48	+	+ +	+
C3a	-	+ +	+	C49	-	+ +	+
C4	-	+ +	+	C50	-	+ +	+
C5	-	+ +	+	C51	+	+ +	+
C6	-	+ +	+	C52	+	+ +	+ +
C7	-	+ +	+	C53	-	+ +	+
C8	-	+ +	+	C54	-	+ +	+
C9	-	+ +	+	C55	-	+ +	+

* + + = coagulation.

\+ = reddening of litmus.

\- = no evidence of growth.

The results presented in this table bring out several facts which seem to show a certain amount of correlation between temperatures at which growth occurs and other significant characters to be discussed in the following sections. Among the nine L.acidophilus cultures from laboratory sources eight grew at 45° C., five at room temperature, and three at 15-20° C. The last three were the only ones among this group which were classed as small and one of the three was the one which failed to grow at 45° C. Moreover, only two of the six large strains showed growth at room temperature, while the small strains caused coagulation at this temperature.

Of the 16 L.acidophilus cultures isolated, two grew at 45° C., eight at room temperature, and eight at 15-20° C., while six showed no growth at any of these temperatures. It should be noted, however, that these six cultures even at 37° C. were extremely slow in their growth. The two cultures which grew at 45° C. were large strains while seven of the eight strains which showed growth at both room temperature and 15-20° C. were classed as small and only one as large.

Of the seven L.bulgaricus cultures six grew well at 45° C., all grew at room temperature, and two at 15-20° C. The growth of these cultures at 45° C. was much more vigor-

ous than that of any of the other cultures reported as showing growth at that temperature, coagulation of the milk occurring in practically every instance in less than 24 hours. The only one which did not grow at 45° C. and the two which grew at 15-20° C. were the only ones in this group which were classed as small.

Among the 54 L.casei cultures, 11 grew at 45° C., 42 at room temperature, and 40 at 15-20° C. There were 12 cultures which failed to grow at any of these temperatures and it is of interest to note that with one exception these were the only strains classed as large in the L.casei group. The exception noted was the only large strain among the 11 which grew at 45° C. and also the only large strain among the 42 which grew at room temperature. One of the small strains which grew at both 45° C. and room temperature failed to grow at 15-20° C. and was the only small strain which failed to do so. As may be noted from the data in the table, growth at 45° C. was not vigorous as only four of the 11 cultures which showed growth caused coagulation. On the other hand growth at room temperature was good, only three of the cultures which grew at this temperature failing to cause coagulation. Growth at 15-20° C. was also good although only four of

the 40 cultures which grew at this temperature caused coagulation in the seven day period.

Of the 86 cultures, 27 grew at 45° C., 62 at room temperature, 53 at 15-20° C.; 18 failed to grow at any of these temperatures. Although it has been stated previously that 37° C. was used for incubation in the routine work with these cultures it should be noted that not all of them grew equally well at that temperature, some of the cultures showing a tendency to slow up considerably if not transferred at frequent intervals. All of those which did not grow at any of the three temperatures just discussed grew rather slowly even at 37° C. and three of them in the group of L.acidophilus cultures isolated never produced enough acid to cause coagulation even after a series of transfers covering more than a year in time.

EFFECT OF SURFACE TENSION ON GROWTH.

The relation of surface tension to the activities of microorganisms has received comparatively little attention. Work along this line has been confined largely to cellular activity as it applies to vital phenomena. In connection with bacteria the principal interest has centered around the antiseptic properties of surface tension depressants or upon the effect which they exert on other materials used as disinfectants. Whether the effect is

due to adsorption of the depressant on the surface of the bacterial cells, thus influencing the permeability of the cell wall to nutrients, or whether due to differences in the structure of the protoplasm is a part of the problem which has not yet been adequately explained. If, however, these differences between bacterial cells do exist and cause fundamentally different reactions on the part of the organisms in the presence of surface tension depressants, even though the mechanism of the action is not positively understood, it may serve as a practical means of differentiation.

Review of Literature.

Larson, Cantwell, and Hartzell (1919) found such characters as spore formation and pellicle formation of B.subtilis to be entirely changed by lowered surface tension.

Larson (1921) reported castor oil soap as the best depressant because it was perfectly clear in water solution and did not hydrolize readily. He also suggested lowered surface tension as favorable to the growth of intestinal organisms.

Ayers, Rupp and Johnson (1923) confirmed the work of Larson on the stability of sodium ricinoleate and its power as a depressant. They also found the composition and initial reaction of the medium, and the nature of the

depressant used important factors determining the amount of growth of certain streptococci. Their results showed streptococci in general to be suppressed at a higher surface tension than intestinal bacteria such as B.coli or B.aerogenes. Because of certain favorable characteristics they suggested sodium glycocholate as the best depressant for the group of organisms studied.

The first investigators to recognize the possible value of lowered surface tension as a means for differentiating between L.acidophilus and L.bulgaricus were Albus and Holm (1925) who reported that with sodium ricinoleate as the depressant the latter failed to grow at a surface tension below 40 dynes while the former was able to grow at a surface tension as low as 36 dynes. The weight-drop method was used in determining surface tensions.

Kopeloff and Beerman (1926), using the same depressant, placed the lower limit of growth for L.bulgaricus at 42 dynes, while L.acidophilus was found to grow abundantly at that and considerably lower surface tensions. In a second brief report of studies on nine cultures of L.acidophilus and six of L.bulgaricus the same investigators (1927) reported that all except one strain of the former were able to grow at a surface tension of three or

more dynes lower than any strain of L.bulgaricus in the presence of sodium ricinoleate. The average critical surface tension with this depressant was 37.9 dynes for L.acidophilus and 43.1 dynes for L.bulgaricus. Two strains of L.acidophilus of proven therapeutic value grew at 35 dynes. Both grew at a lower surface tension with sodium oleate than with "equivalent concentrations" of sodium ricinoleate.

Frobisher (1926) found sodium oleate to be the most useful depressant as it was comparatively inert to bacterial metabolism while sodium glycocholate was found to be more subject to metabolism and more likely to be hydrolyzed by acids.

Methods.

In this investigation all surface tension measurements were made by the ring method with the Cenco-DuNoüy tensiometer, the material tested being at 25° C. A small amount of the medium to be tested was placed in a two-inch cover glass which had been cleaned in hot cleaning solution, rinsed in hot water, then rinsed in distilled water, and finally wiped dry with a clean towel and lens paper. Since trials had shown that the surface tension changed for a minute or two before reaching comparative equilibrium,

the determination was not made until two minutes after the sample had been placed in the cover glass. The surface tension values presented represent the average of several determinations, although duplicates ordinarily checked very closely when the preliminary wait of two minutes was made.

The instrument was standardized by the absolute method and was checked in this manner and with distilled water several times during the course of the experiments. The value of g at Ames is 980.26. The formula r equals $\frac{wg}{2c}$ then becomes r equals $\frac{.700 \times 980.26}{2 \times 3.99}$ or 85.77. This value was used in the standardization.

The media used were medium X and medium Y of Albus and Holm (1926); medium M, which was made up exactly like medium X except that the lactose was replaced with maltose; beef infusion bouillon; and whey peptone broth. In all cases 0.5 cc. of a 5% alcoholic solution of brom cresol purple per liter was used as an indicator and any change in color as compared to an uninoculated check tube accepted as evidence of growth. The use of an indicator has the added advantage of counterbalancing the difficulties usually encountered in detecting growth rapidly in a medium which sterilization causes to become cloudy after

a change in reaction or after the addition of some such material as those used as surface tension depressants.

The bouillon was prepared by adding 1% lactose to unneutralized beef infusion containing 1% Difco peptone and 0.5% NaCl. The whey peptone broth was made by adding 2% Difco peptone, and 0.5% NaCl to whey secured by coagulating fresh skim milk with rennet, heating to shrink the curd and then straining through cheese cloth. Although the latter medium formed a heavy precipitate on sterilization, the indicator obviated errors in detecting growth.

In several preliminary trials the method of adding a solution of the depressant to a tube of medium was found to be unsatisfactory so the method adopted for the rest of the work was that of adding the depressant directly to the medium until it was found by trial that approximately the desired surface tension had been reached. As a rule the surface tension was slightly higher after sterilization so it was adjusted to a point slightly lower than was desired in the finished medium. The material was then tubed and sterilized in the autoclave and the surface tension again checked before inoculation.

The sodium ricinoleate used as a surface tension depressant was prepared in this laboratory after the method of Halvorson (1925).

Other depressants used were commercial preparations.

The reaction of the medium was determined before the final adjustment of the surface tension and checked before tubing to allow for any necessary readjustments. All pH determinations were made by the colorimetric method as outlined by Brown (1924).

All of the cultures were carried in litmus milk and in all cases the special medium was inoculated from litmus milk cultures which had been incubated at 37° C. for 48 hours; the same sized loop was used throughout for all transfers.

Results Obtained.

The effect of lowered surface tension on the growth of 27 cultures in medium X and medium Y using sodium taurocholate and sodium glycocholate as depressants is shown by the data presented in Table III.

The pH of medium X without depressant was 5.8 and the pH values of two lots of this medium with 0.2 and 0.5 grams of sodium taurocholate per 100 cc. were 5.8 and 6.0 respectively. The surface tensions of these three lots of this medium were 51.0, 42.1, and 38.4 dynes respectively. The pH values of two lots of the same medium with the

TABLE II

Effect of lowered surface tension on medium Y with sodium taurocholate and depressants.

Cult.	Medium X				
	no depress: s.t.51.0*	with sodium taurocholate s.t. 42.1	with sodium taurocholate s.t. 38.4	with sodium glycocholate s.t. 39.5	with sodium glycocholate s.t. 38.5
A1	+ +	+ +	+ +	+	-
A3	+	+ +	+	+	-
A3a	+	+ +	+	+	-
A3b	+	+ +	+	+	-
A4	+	+	+	+	-
A5	+	+ +	+ +	+	-
A6	+	+	+	-	-
A7	+	+	+	+	-
A8	+	+ +	+ +	+	-
A9	+ +	+ +	+ +	+	-
B1	+ +	+ +	+ +	+	-
B2	+	+	+	-	-
B3	+ +	+ +	+	+	-
B5	+	+	+	-	-
S1	+ +	+ +	+ +	+	-
S5	+ +	+ +	+ +	+	-
S6	+	+	+	+	+
S7	+ +	+ +	+ +	+	-
S8c	+	+ +	+	-	-
S11	+	-	-	-	-
S12	+ +	+ +	+ +	+	-
S15	+ +	+ +	+ +	+	-
S15a	+ +	+ +	+ +	+	-
C1	+ +	+ +	+ +	+	-
C2	+ +	+ +	+ +	+	+
C3	+	+ +	+	+	-
C3a	+ +	+ +	+	+	-

* in this and the following tables on surf

s.t. = surface tension in dyn
+ + = growth in 1 - 3 days.
+ = growth in 4 - 7 days.
- = no growth in 7 days.

E III

on on growth in medium X and
te and sodium glycocholate as

	Medium Y			
	no depress:	with sodium taurocholate		with sodium glycocholate
5	s.t. 52.0	s.t. 47.0	s.t. 45.0	s.t. 45.5 : s.t. 42.0
	+ +	+ +	+ +	+ + + +
	+	+	+	+ +
	+	+	+	+ -
	+	+	+	+ -
	+	-	+	- -
	+	+	+	+ -
	-	-	-	- -
	+	+ +	+	+ +
	+	+	+	+ +
	+ +	+ +	+ +	+ + + +
	+ +	+ +	+ +	+ + + +
	+	-	-	- -
	+ +	+	+	+ -
	+	-	-	- -
	+ +	+ +	+ +	+ + + +
	+ +	+ +	+ +	+ + + +
	+	+	+	+ +
	+ +	+ +	+ +	+ + + +
	+	-	-	- -
	-	-	-	- -
	+ +	+ +	+ +	+ + + +
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	+ +	+ +	+ +	+ + + +
	+ +	+ +	+ +	+ + + +
	+ +	+ +	+ +	+ + + +
	+	+	+	+ -
	+ +	+ +	+ +	+ + + +

n surface tension

in dynes
days.
days.
days.

same amounts of sodium glycocholate were 6.0 and 5.9, and the surface tensions 39.5 and 38.5 dynes respectively.

All of the 27 cultures grew in medium X without depressant, and only one of these failed to grow in this medium with the surface tensions reduced to 42.1 and 38.4 dynes with sodium taurocholate. The culture which failed to grow was one of those classed as large in the group of L.acidophilus cultures isolated. With sodium glycocholate as the depressant in this medium five cultures failed to grow at 39.5 dynes, and 25 failed to grow at 38.5 dynes. Of the five cultures which did not grow at the higher surface tension one was in the laboratory L.acidophilus group, two in the L.bulgaricus group, and two in the isolated L.acidophilus group. These were all classed as large. The two cultures which grew at the lower surface tension were an L.casei and an isolated L.acidophilus both of which were classed as small.

The pH of medium Y without depressant was 5.5, and the pH values of two lots of this medium with 0.1 and 0.2 grams of sodium taurocholate per 100 cc. were 5.8 in both cases. The surface tensions in the three lots of this medium were 52.0, 47.0, and 45.0 dynes respectively. The pH values of two lots of the same medium with the same amounts of sodium glycocholate were also

5.8 in both cases, and the surface tensions were 45.5 and 42.0 dynes respectively.

Twenty-five of the 27 cultures grew in medium Y without depressant. One of the two cultures which failed to grow in this medium without depressant did grow in it during later trials. Of the 25 cultures which grew, four failed to grow with the surface tension depressed to 47.0 dynes with sodium taurocholate. One of these was in the laboratory L. acidophilus group, two in the L.bulgaricus group, and one in the isolated L.acidophilus group - all were classed as large. With the surface tension of 45.0 dynes the same cultures failed to grow with the exception of the L.acidophilus culture from a laboratory source. With sodium glycocholate as the depressant in this medium the same four cultures failed to grow at a surface tension of 45.5 dynes, and nine, including these four, failed to grow at 42.0 dynes. Of the five additional cultures one was in the L.bulgaricus group, one in the L.casei group, and the other three in the laboratory L.acidophilus group. Two of the latter were cultures A3a and A3b, although culture A3 from which they were obtained did grow. The L.casei culture was classed as small, and the other four cultures as large.

There are several comparisons which can readily be made from the results of these trials. Marked difference is evident in the growth of these cultures at a surface tension of 38.4 dynes in medium X with sodium taurocholate as the depressant and in the same medium depressed to 38.5 dynes with sodium glycocholate although the same amounts of depressant were used in both cases. The results obtained with medium X depressed to 42.1 dynes with sodium taurocholate are also quite different from those obtained with medium Y depressed to 42.0 dynes with sodium glycocholate. In the former case only one culture did not grow, whereas in the latter, nine cultures which had grown in the medium without depressant failed to grow at the reduced surface tension. In these instances also the same amounts of depressants were used.

Because of the apparent inconsistencies noted and because of the fact that such large amounts of these depressants were necessary to appreciably lower the surface tensions of these media, it seemed advisable to use a depressant having better properties. Sodium oleate was then used since various investigators had suggested it as a valuable material combining strong depressing power with other good qualities.

The results of two trials in which sodium oleate

was used as the depressant are presented in Table IV.

The pH of medium X without depressant was 6.4 and the pH values of two lots of this medium with 0.17 and 0.23 grams of sodium oleate per 100 cc. were 6.7 and 6.8 respectively. The surface tensions of these three lots of this medium were 50.0, 38.5, and 35.5 dynes respectively.

All of the 27 cultures grew in the medium without depressant and in that depressed to 38.5 dynes; four failed to grow at 35.5 dynes. These four cultures grew in the same medium depressed to 38.4 dynes with sodium taurocholate but were among the five which failed to grow at 39.5 dynes with sodium glycocholate as the depressant. It was indicated in connection with the previous trial that one of these cultures was in the laboratory L.acidophilus group, two in the L.bulgaricus group, and one in the isolated L.acidophilus group and that all were classed as large.

The pH of medium Y without depressant was 6.6 and the pH values of the two lots of this medium with 0.2 and 0.25 grams of sodium oleate per 100 cc. were 7.0 and 6.7 respectively. The surface tensions of these three lots of this medium were 51.5, 43.0 and 36.8 dynes respectively.

TABLE IV

Effect of lowered surface tension on growth in medium X and medium Y with sodium oleate as depressant.

Cult.	Medium X		Medium Y	
	without depressant	with depressant	without depressant	with depressant
	s.t. 50.0	s.t. 38.5 s.t. 35.5	s.t. 51.5	s.t. 43.0 s.t. 36.8
A1	+ +	+ + + +	+ +	+ + + +
A3	+ +	+ + + +	+ +	+ + + +
A3a	+ +	+ + + +	+ +	+ + + +
A3b	+ +	+ + + +	+ +	+ + + +
A4	+ +	+ + + +	+ +	-
A5	+ +	+ + + +	+	-
A6	+ +	+ + -	+ +	+ -
A7	+ +	+ + + +	+ +	+ + + +
A8	+ +	+ + + +	+ +	+ + + +
A9	+ +	+ + + +	+ +	+ + + +
B1	+ +	+ + + +	+ +	+ + + +
B2	+ +	+ -	+	- -
B3	+ +	+ + + +	+ +	+ + -
B5	+ +	+ -	+	- -
S1	+ +	+ + + +	+ +	+ + + +
S5	+ +	+ + + +	+ +	+ + + +
S6	+ +	+ + + +	+ +	+ + +
S7	+ +	+ + + +	+ +	+ + + +
S8c	+ +	+ + + +	+	+ + + +
S11	+	+ -	-	- -
S12	+ +	+ + + +	+ +	+ + + +
S15	+ +	+ + + +	+ +	+ + + +
S15a	+ +	+ + + +	+ +	+ + + +
C1	+ +	+ + + +	+ +	+ + + +
C2	+ +	+ + + +	+ +	+ + + +
C3	+ +	+ + + +	+ +	+ + + +
C3a	+ +	+ + + +	+ +	+ + + +

Twenty-six of the 27 cultures grew in medium Y without depressant. The culture which failed to grow was one of the two which failed to grow in this medium in the previous trial. It did not grow in this medium with sodium oleate added. Two L.bulgaricus cultures failed to grow at a surface tension of 43.0 dynes, and six cultures, including these two, failed to grow at 36.8 dynes. One of the four additional cultures was in the L.bulgaricus group, and three in the laboratory/L.acidophilus group. These six cultures were among those which did not grow in the same medium at 42.0 dynes with sodium glycocholate as the depressant, and all were classed as large.

It is noticeable from these data that most of the cultures were able to grow in these two media at lower surface tensions with sodium oleate as the depressant than with either sodium taurocholate or sodium glycocholate. The cultures which failed to grow at the lower surface tensions in this trial were among those which were inhibited by reduced surface tensions with sodium taurocholate and sodium glycocholate.

The work of several investigators and preliminary trials made in the present work indicated that sodium ricinoleate was a more satisfactory depressant than any

previously used. Much smaller amounts were necessary to secure the desired surface tensions and there apparently was less change in the surface tension of a medium during steriliation. It was accordingly used in the rest of the surface tension studies.

The data obtained from two trials in which medium X and sodium ricinoleate were used are presented in Table V.

The pH values of the medium without depressant in the two trials were 6.7 and 6.2. The pH values of the two lots of this medium with 0.04 and 0.008 grams of sodium ricinoleate per 100 cc. were likewise 6.7 and 6.2. The surface tensions of the medium without depressant in each trial were 50.5 and 49.0 dynes respectively, and with depressant 37.4 and 40.0 dynes respectively.

All of the cultures tested grew in medium X without depressant in both trials and at 40.0 dynes. Twenty-three of the 82 cultures did not grow at a surface tension of 37.4 dynes. Among these 23 cultures there were six in the laboratory L.acidophilus group, three of which were classed as small and three as large; five in the L.bulgaricus group, all of which were classed as large; five in the isolated L.acidophilus group, one of which was classed as small and four as large; and seven in the

T A B L

Effect of lowered surface ten
sodium ricinoleate as depress

Cult.	Trial-1		Trial-2	
	without depressant s.t. 50.5	with depressant s.t. 37.4	without depressant s.t. 49.0	with depressant s.t. 40.0
A1	+ +	+ +	+ +	+ +
A3	+ +	-	+ +	+ +
A3a	+ +	-	+ +	+ +
A3b	+ +	-	+ +	+ +
A4	+ +	-	+ +	+ +
A5	+ +	-	+ +	+ +
A6	+ +	-	+ +	+
A7	+ +	+	+ +	+ +
A8	+ +	+	+ +	+ +
A9	+ +	+ +	+ +	+ +
A10	+ +	+ +		
B1	+ +	+ +	+ +	+ +
B2	+ +	-	+ +	+
B3	+ +	-	+ +	+ +
B5	+ +	-	+ +	+
B6	+ +	-	+ +	+ +
B7	+ +	+	+ +	+ +
B8	+ +	-	+ +	+
S1	+ +	+ +	+ +	+ +
S5	+ +	-	+ +	+ +
S6	+ +	+	+ +	+ +
S7	+ +	+ +	+ +	+ +
S8a	+	-		
S8b	+	-		
S8c	+ +	+	+ +	+ +
S9a	+ +	-		
S9b	+ +	+		
S10	+ +	+ +		
S11	+	-	+ +	+
S12	+ +	+ +	+ +	+ +
S15	+ +	+ +	+ +	+ +
S15a	+ +	+ +	+ +	+ +
C1	+ +	+	+ +	+ +
C2	+ +	+ +	+ +	+ +
C3	+ +	-	+ +	+ +
C3a	+ +	+ +	+ +	+ +
C4	+ +	+	+ +	+ +
C5	+ +	+	+ +	+ +
C6	+ +	+	+ +	+ +
C7	+ +	+ +	+ +	+ +
C8	+ +	+ +	+ +	+ +

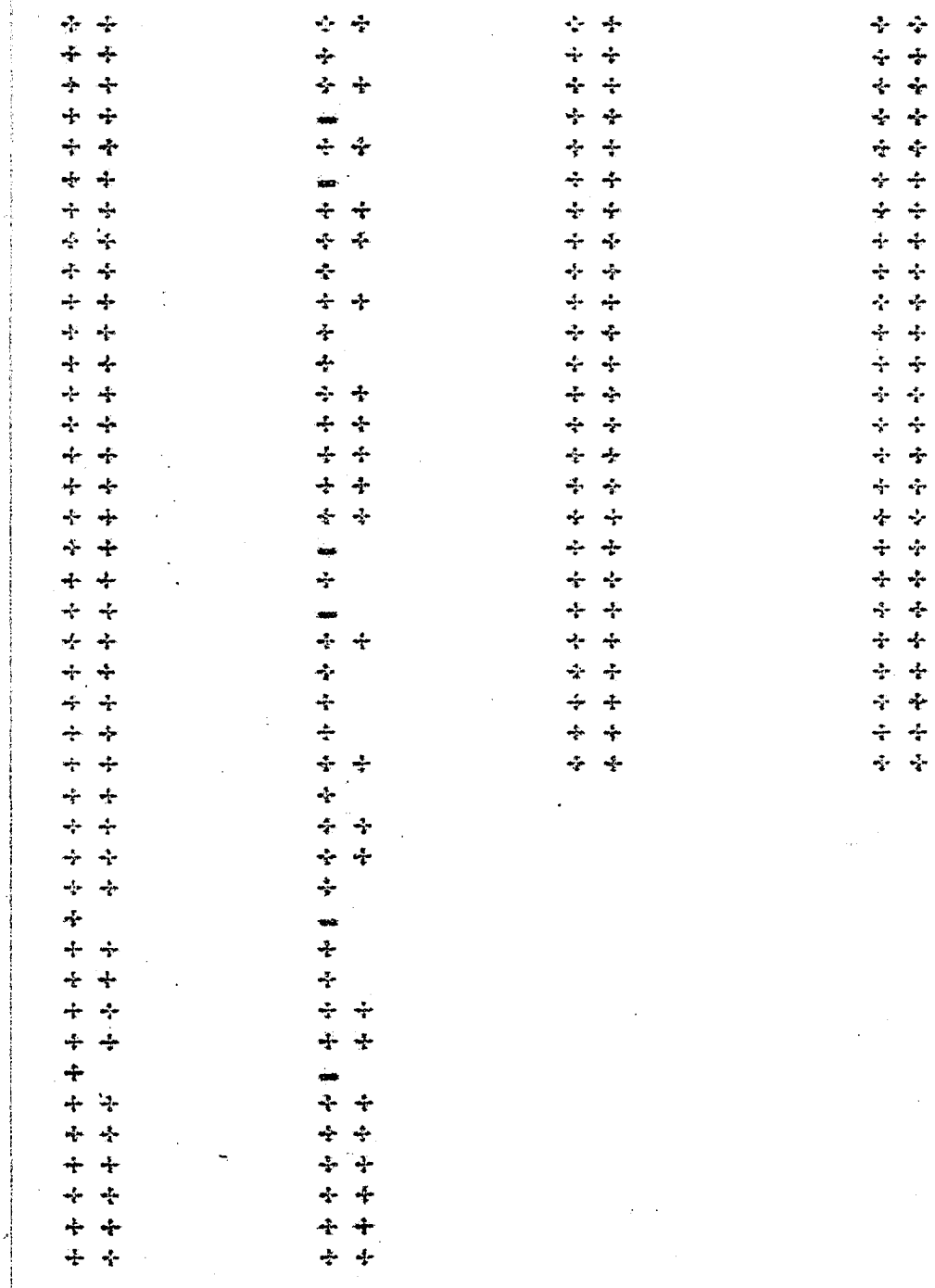
TABLE V

lowered surface tension on growth in medium X with cinoleate as depressant.

Trial-2			Trial-1		
with depressant s.t. 40.0		Cult.	without depressant s.t. 50.5	with depressant s.t. 57.4	without depressant s.t. 49.0
+ +		C9	+ +	+ +	+ +
+ +		C10	+ +	+	+ +
+ +		C12	+ +	+ +	+ +
+ +		C14	+ +	-	+ +
+ +		C17	+ +	+ +	+ +
+ +		C18	+ +	-	+ +
+		C19	+ +	+ +	+ +
+ +		C20	+ +	+ +	+ +
+ +		C20a	+ +	+	+ +
+ +		C21	+ +	+ +	+ +
		C22	+ +	+	+ +
+ +		C22a	+ +	+	+ +
+		C23	+ +	+ +	+ +
+ +		C25	+ +	+ +	+ +
+		C27	+ +	+ +	+ +
+ +		C28	+ +	+ +	+ +
+ +		C29	+ +	+ +	+ +
+		C29a	+ +	-	+ +
+ +		C30	+ +	+	+ +
+ +		C31	+ +	-	+ +
+ +		C32	+ +	+ +	+ +
+ +		C32a	+ +	+	+ +
		C33	+ +	+	+ +
		C34	+ +	+	+ +
+ +		C36	+ +	+ +	+ +
		C40	+ +	+	
		C41	+ +	+ +	
		C42	+ +	+ +	
+		C43	+ +	+	
+ +		C44	+	-	
+ +		C45	+ +	+	
+ +		C46	+ +	+	
+ +		C47	+ +	+ +	
+ +		C48	+ +	+ +	
+ +		C49	+	-	
+ +		C50	+ +	+ +	
+ +		C51	+ +	+ +	
+ +		C52	+ +	+ +	
+ +		C53	+ +	+ +	
+ +		C54	+ +	+ +	
+ +		C55	+ +	+ +	

growth in medium X with

Trial-1		Trial-2	
Without depressant	With depressant	Without depressant	With depressant
s.t. 50.5	s.t. 37.4	s.t. 49.0	s.t. 40.0



L.casei group, three of which were classed as small and four as large.

As in the previous trials there appears to be some correlation between the various characters of the cultures failing to grow at reduced surface tensions, particularly in size.

The data obtained from one trial in which medium Y and sodium ricinoleate were used are presented in Table VI.

The pH value of this medium without depressant was 6.6 and with 0.012 grams of sodium ricinoleate per 100 cc. it was 6.4. The surface tensions of these two lots of the medium were 50.0 and 39.4 dynes respectively.

Of the 60 cultures tested, one failed to grow in the medium without depressant and also at the reduced surface tension. This was the same culture which failed to grow in this medium in previous trials.

In order to check the ability of the cultures to ferment maltose one trial was made with medium M which was of the same composition as medium X except that maltose was substituted for lactose. The data obtained from this trial are presented in Table VII.

The pH value of the medium both with and without sodium ricinoleate was 6.6. The surface tension of the

TABLE VI

Effect of lowered surface tension on growth in medium Y with sodium ricinoleate as depressant.

Cult.	Without depressant s.t. 50.0	With depressant s.t. 39.4	Cult.	Without depressant s.t. 50.0	With depressant s.t. 39.4
A1	+ +	+ +	C4	+ +	+ +
A3	+ +	+ +	C5	+ +	+ +
A3a	+ +	+ +	C6	+ +	+ +
A3b	+ +	+ +	C7	+ +	+ +
A4	+ +	+ +	C8	+ +	+ +
A5	+ +	+ +	C9	+ +	+ +
A6	+ +	+	C10	+ +	+ +
A7	+ +	+ +	C12	+ +	+ +
A8	+ +	+ +	C14	+ +	+ +
A9	+ +	+ +	C17	+ +	+ +
B1	+ +	+ +	C18	+ +	+ +
B2	+ +	+	C19	+ +	+ +
B3	+ +	+ +	C20	+ +	+ +
B5	+ +	+	C20a	+ +	+ +
B6	+ +	+ +	C21	+ +	+ +
B7	+ +	+ +	C22	+ +	+ +
B8	+ +	+	C22a	+ +	+ +
S1	+ +	+ +	C23	+ +	+ +
S5	+ +	+ +	C25	+ +	+ +
S6	+ +	+ +	C27	+ +	+ +
S7	+ +	+ +	C28	+ +	+ +
S8c	+ +	+ +	C29	+ +	+ +
S11	-	-	C29a	+ +	+ +
S12	+ +	+ +	C30	+ +	+ +
S15	+ +	+ +	C31	+ +	+ +
S15a	+ +	+ +	C32	+ +	+ +
C1	+ +	+ +	C32a	+ +	+ +
C2	+ +	+ +	C33	+ +	+ +
C3	+ +	+ +	C34	+ +	+ +
C3a	+ +	+ +	C36	+ +	+ +

TABLE VII

Effect of lowered surface tension on growth in medium M with sodium ricinoleate as depressant.

Cult.	Without depressant s.t. 51.0	With depressant s.t. 39.0	Cult.	Without depressant s.t. 51.0	With depressant s.t. 39.0
A1	+ +	+ +	C10	+ +	+ +
A3	+ +	+	C12	+ +	+ +
A3a	+ +	+ +	C14	+ +	+ +
A3b	+ +	+ +	C17	+ +	+ +
A4	+ +	-	C18	+ +	+ +
A5	+ +	-	C19	+ +	+ +
A6	-	-	C20	+ +	+ +
A7	+ +	+ +	C20a	+ +	+ +
A8	+ +	+ +	C21	+ +	+ +
A9	+ +	+ +	C22	+ +	+ +
B1	+ +	+ +	C23	+ +	+ +
B2	-	-	C25	+ +	+ +
B3	+ +	-	C27	+ +	+ +
B5	-	-	C28	+ +	+ +
B6	+ +	-	C29	+ +	+ +
B7	+ +	+ +	C29a	+ +	+ +
B8	-	-	C30	+ +	+ +
S1	+ +	+ +	C31	+ +	+
S5	+ +	+ +	C32	+ +	+ +
S6	+ +	+ +	C32a	+ +	+ +
S7	+ +	+ +	C33	+ +	+ +
S8c	+ +	+ +	C34	+ +	+
S11	+	-	C36	+ +	+
S12	+ +	+ +	C40	+	+
S15	+ +	+ +	C41	+ +	+ +
S15a	+ +	+ +	C42	+ +	+ +
C1	+ +	+ +	C43	+ +	+
C2	+ +	+ +	C44	+	+
C3	+ +	+ +	C45	+	+
C3a	+ +	+ +	C46	+ +	+ +
C4	+ +	+ +	C47	+ +	+ +
C5	+ +	+	C48	+ +	+

A8	+ +	+ +	C21	+ +	+ +
A9	+ +	+ +	C22	+ +	+ +
B1	+ +	+ +	C23	+ +	+ +
B2	-	-	C25	+ +	+ +
B3	+ +	-	C27	+ +	+ +
B5	-	-	C28	+ +	+ +
B6	+ +	-	C29	+ +	+ +
B7	+ +	+ +	C29a	+ +	+ +
B8	-	-	C30	+ +	+ +
S1	+ +	+ +	C31	+ +	+
S5	+ +	+ +	C32	+ +	+ +
S6	+ +	+ +	C32a	+ +	+ +
S7	+ +	+ +	C33	+ +	+ +
S8c	+ +	+ +	C34	+ +	+
S11	+	-	C36	+ +	+
S12	+ +	+ +	C40	+	+
S15	+ +	+ +	C41	+ +	+ +
S15a	+ +	+ +	C42	+ +	+ +
C1	+ +	+ +	C43	+ +	+
C2	+ +	+ +	C44	+	+
C3	+ +	+ +	C45	+	+
C3a	+ +	+ +	C46	+ +	+ +
C4	+ +	+ +	C47	+ +	+ +
C5	+ +	+	C48	+ +	+
C6	+ +	+ +	C49	+	+
C7	+ +	+ +	C52	+ +	+ +
C8	+ +	+ +	C53	+ +	+
C9	+ +	+ +	C55	+ +	+

lot without depressant was 51.0 dynes, while that of the lot with 0.023 grams of the depressant per 100 cc. was 39.0 dynes.

Four of the 72 cultures tested failed to grow in the medium without depressant. Three of these were in the L.bulgaricus group and one in the group of L.acidophilus cultures from laboratory sources; all were classed as large. Nine cultures, including these four, did not grow at the reduced surface tension. Of the nine, three were L.acidophilus from laboratories, five were L.bulgaricus, and one an isolated L.acidophilus culture; all were classed as large. The L.bulgaricus cultures were all of the large ones in that group. The isolated L.acidophilus culture was the one which had previously failed to grow under reduced surface tensions.

One surface tension trial was made with beef infusion bouillon. The data obtained are presented in Table VIII.

The pH values of the bouillon without depressant and that of the lot with 6.25 milligrams of sodium ricinoleate per 100 cc. were both 6.6. The surface tensions were 46.0 and 39.6 dynes respectively.

Of the 60 cultures tested, two failed to grow in the medium without depressant; the growth of many of the

T A B L E VIII

Effect of lowered surface tension on growth in beef infusion bouillon with sodium ricinoleate as depressant.

Cult.	Without depressant s.t. 46.0	With depressant s.t. 39.6	Cult.	Without depressant s.t. 46.0	With depressant s.t. 39.6
A1	+ +	-	C4	+ +	+ +
A3	+ +	+	C5	+ +	+ +
A3a	+	+	C6	+	+ +
A3b	+ +	+	C7	+ +	+ +
A4	+	-	C8	+ +	+ +
A5	+ +	-	C9	+ +	+ +
A6	+ +	-	C10	+ +	+ +
A7	+	-	C12	+ +	+ +
A8	+	-	C14	+	-
A9	+ +	+ +	C17	+ +	+ +
B1	+ +	+ +	C18	-	-
B2	+	+	C19	+ +	+ +
B3	+ +	+	C20	+ +	+ +
B5	+	-	C20a	+	-
B6	+ +	-	C21	+ +	+ +
B7	+ +	+ +	C22	+ +	+ +
B8	+	+	C22a	+ +	+ +
S1	+ +	+ +	C23	+ +	+ +
S5	+ +	+ +	C25	+ +	+ +
S6	+ +	+ +	C27	+ +	+ +
S7	+ +	+ +	C28	+ +	+ +
S8c	+	+ +	C29	+ +	+ +
S11	-	-	C29a	+	-
S12	+ +	+ +	C30	+	-
S15	+ +	+ +	C31	+	+
S15a	+ +	+ +	C32	+ +	+ +
C1	+ +	+ +	C32a	+ +	-
C2	+ +	+ +	C33	+	-
C3	+ +	+ +	C34	+	-
C3a	+ +	+ +	C36	+ +	+ +

other cultures was not vigorous. One of the two which did not grow was the isolated L.acidophilus culture which had previously failed to grow in medium Y, and the other was a slow growing L.casei strain; both cultures were classed as large. Seventeen cultures, including the two just discussed, did not grow under the reduced surface tension. Of these, six were laboratory L.acidophilus cultures, two L.bulgaricus cultures, one the L.acidophilus strain discussed above, and eight L.casei cultures. With the exception of one laboratory L.acidophilus strain, these were all classed as large. The one small culture did grow, however, in all other trials with reduced surface tensions except once when sodium glycocholate was used. The large strains among the L.acidophilus and L.bulgaricus groups were practically all the same ones which had shown tendencies of being inhibited in other trials with reduced surface tensions.

Whey peptone broth was found to be an excellent medium for the growth of the organisms studied. With one or two exceptions all of the cultures grew vigorously in it. The data obtained in two surface tension trials with this medium are presented in Table IX.

The pH of this medium in trial 1, both with and without depressant, was 6.5. The surface tension of the

TABLE

Effect of lowered surface tension on
with sodium ricinoleate as depressant

Cult.	Trial-1		Trial-2		Cult.
	without depressant	with depressant	without depressant	with depressant	
	s.t. 43.1	s.t. 37.3	s.t. 48.0	s.t. 39.0	
A1	+ +	+	+ +	+ +	C10
A3	+ +	+	+ +	+ +	C12
A3a	+ +	+	+ +	+ +	C13
A3b	+ +	+	+ +	+ +	C14
A4	+ +	-	+ +	-	C16
A5	+ +	-	+ +	+	C17
A6	+ +	-	+ +	-	C18
A7	+ +	+	+ +	+ +	C19
A8	+ +	+ +	+ +	+ +	C20
A9	+ +	+	+ +	+ +	C20
A10	+ +	+	+ +	+ +	C21
B1	+ +	+	+ +	+ +	C22
B2	+ +	-	+ +	-	C22
B3	+ +	-	+ +	+ +	C23
B5	+ +	-	+ +	-	C24
B6	+ +	-	+ +	-	C25
B7	+ +	+	+ +	+ +	C26
B8	+ +	-	+ +	-	C27
S1	+ +	-	+ +	+ +	C28
S5	+ +	-	+ +	+ +	C29
S6	+ +	-	+ +	+ +	C29
S7	+ +	-	+ +	+ +	C30
S8a	+ +	-	+ +	+	C31
S8b	+ +	-	+ +	-	C32
S8c	+ +	+	+ +	+ +	C32
S9a	+ +	-	+ +	+ +	C33
S9b	+ +	+ +	+ +	+ +	C34
S10	+ +	+	+ +	+	C35
S11	+ +	-	+ +	+ +	C40
S12	+ +	-	+ +	+ +	C41
S15	+ +	+	+ +	+ +	C42
S15a	+ +	-	+ +	+ +	C43
S16	+ +	+	+ +	+ +	C44
S17			+ +	+ +	C45
C1	+ +	-	+ +	+ +	C46
C2	+ +	-	+ +	+ +	C47
C3	+ +	-	+ +	+ +	C48
C3a	+ +	-	+ +	+ +	C49
C4	+ +	-	+ +	+ +	C50
C5	+ +	-	+ +	+ +	C51
C6	+ +	+ +			C52
C7	+ +	+	+ +	+ +	C53
C8	+ +	-	+ +	+ +	C54
C9	+ +	-	+ +	+ +	C55

B L E IX

sion on growth in whey peptone broth
pressant.

Cult.	Trial-1 without depressant s.t. 43.1	Trial-1 with depressant s.t. 37.5	Trial-2 without depressant s.t. 48.0	Trial-2 with depressant s.t. 39.0
C10	+ +	+	+ +	+ +
C12	+ +	-	+ +	+ +
C13	+ +	-	+ +	+ +
C14	+ +	+	+ +	+
C16	+ +	+	-	+
C17	+ +	-	+ +	+ +
C18	+ +	-	+ +	+ +
C19	+ +	-	+ +	+ +
C20	+ +	-	+ +	+ +
C20a	+ +	-	+ +	+ +
C21	+ +	-	+ +	+ +
C22	+ +	+	+ +	+ +
C22a	+ +	+	+ +	+ +
C23	+ +	-	+ +	+ +
C24	+ +	+	+ +	+ +
C25	+ +	-	+ +	+ +
C26	+ +	+	+ +	-
C27	+ +	-	+ +	+ +
C28	+ +	-	+ +	+ +
C29	+ +	-	+ +	+ +
C29a	+ +	+	+ +	+
C30	+ +	+	+ +	+
C31	+ +	+ +	+ +	+
C32	+ +	-	+ +	+ +
C32a	+ +	-	+ +	+
C33	+ +	+	+ +	-
C34	+ +	+	+ +	+
C36	+ +	-	+ +	+ +
C40	+ +	+	+ +	+ +
C41	+ +	-	+ +	+ +
C42	+ +	-	+ +	+ +
C43	+ +	-	+ +	+ +
C44	+ +	-	+ +	+ +
C45	+ +	-	+ +	+ +
C46	+ +	-	+ +	+ +
C47	+ +	-	+ +	+ +
C48	+ +	-	+ +	+ +
C49	+ +	-	+ +	+ +
C50	+ +	-	+ +	+ +
C51	+ +	-	+ +	+ +
C52	+ +	-	+ +	+ +
C53	+ +	-	+ +	+ +
C54	+ +	-	+ +	+ +
C55	+ +	-	+ +	+ +

medium without depressant was 43.1 dynes, and that of the lot with 0.025 grams of sodium ricinoleate per 100 cc. was 37.5 dynes.

The 87 cultures tested in trial 1 all grew well in the medium without depressant while 58 failed to grow at the reduced surface tension. Among the L.acidophilus and L.bulgaricus cultures the ones which did not grow were in general those which consistently exhibited the same characteristics in the previous surface tension studies. It is of interest to note that the three small strains in the laboratory L.acidophilus group were among six which grew, and the two small L.bulgaricus cultures were the only ones in that group which grew. There appeared to be no correlation in the characters of the cultures which did or did not grow in the L.casei and isolated L.acidophilus groups.

In trial 2 the pH of the medium both with and without depressant was 6.7. The surface tension of the medium without depressant was 48.0 dynes, and that of the lot with 0.04 grams of depressant per 100 cc. was 39.0 dynes. The apparent discrepancy in the amounts of depressant necessary in trial 1 and trial 2 may be accounted for in part at least by the difference between the surface tensions of the basic media.

Of the 87 cultures tested in trial 2, one failed to grow in the medium without sodium ricinoleate although it grew in the medium with the depressant. Nine cultures failed to grow in the medium at a surface tension of 39.0 dynes. Of these, two were in the laboratory L.acidophilus group, four in the L.bulgaricus group, one in the isolated L.acidophilus group, and two in the L.casei group; these cultures were all classed as large.

CHARACTER AND AMOUNT OF ACID PRODUCED.

The total amount of acid and the isomeric form of lactic acid produced in milk by the lactobacilli has for many years been a question over which there has been a great deal of controversy among those who have studied this character of the organisms. Any regular difference existing between the various species in this regard might be considered a valid character upon which to base decisions as to the proper identification of an organism because it is one which is constant and which can be determined accurately. With certain other lactic acid formers, such as Streptococcus lactis, this is a character which has been definitely established as constant and is accepted as one point which it is perfectly safe to use in the identification of members of the species.

The amount of volatile acid produced, while possibly of less significance from the standpoint of separating these organisms than the isomeric modification of lactic acid formed, is of considerable importance in other respects. The characteristic flavor of certain types of hard cheese has been ascribed in large measure to the volatile acids produced during ripening. That the L.casei types play an important part in this process is a generally accepted view, and part of their effect at least may be due to the volatile acid which they produce.

A comparison of the total acid and volatile acid production of L.casei, L.acidophilus, and L.bulgaricus as well as a study of the type of acid formed by cultures of L.acidophilus seemed to be advisable because of the possible value which these characters might have in the proper identification and classification of members of these species.

Review of Literature.

One of the earliest reports on the type of lactic acid produced by the lactobacilli is that of Orla-Jensen (1904) who stated that B.casei epsilon may produce over 2.7% of inactive lactic acid in milk. The zinc lactate prepared from the acid contained 18.2% of H_2O of crystal-

lization and 27.5% of ZnO; it showed no rotatory properties. The volatile acid produced by this organism was found to be somewhat more than that produced by Bacterium lactis acidi. Most of the volatile acid was found to be acetic with only small amounts of formic and propionic.

Grigoroff (1905) stated that his Bacillus A. (B.bulgaricus) produced the inactive form of lactic acid.

Bertrand and Weisweiller (1906) concluded from a study of cultures of B.bulgaricus supplied by Metchnikoff that the lactic acid produced by the organisms was a mixture of the laevo and dextro forms, with the latter predominating. The acid was reported as being practically all lactic with about 3% succinic, and probably some formic and acetic.

The statement of Luerssen and Kühn (1908) that the whey from milk fermented by B.bulgaricus turned polarized light to the right is of questionable value.

Heinemann and Hefferan (1909) found the acid produced by B.bulgaricus to be inactive without a trace of the active form being left in the mother liquor after removing the zinc salts. They found 5.8-6.1% of the total acid produced to be volatile. The nature of the volatile acids was not determined.

Bertrand and Duchacek (1909) reported the production of equal amounts of laevo and dextro acids by B.bulgaricus from glucose, galactose, levulose, and mannose but for some unexplained reason a relative excess of the latter in milk. They also stated that small amounts of succinic, formic, and acetic acids were produced.

White and Avery (1910) found their high acid type (Type A) from yoghurt, mazun, and leben formed either inactive or laevo acid, while their low acid type (Type B) formed principally laevo, more rarely inactive or dextro. All of their cultures produced small amounts of volatile acids, the exact amount and nature of which were not determined.

Varying results were obtained by Currie (1911) who found that high acid strains of B.bulgaricus from various sources formed an excess of dextro acid. Two cultures from milk soured at 38° C. formed inactive and laevo; two from cheddar cheese formed dextro and inactive; two from cheddar cheese formed inactive and laevo; one from the same source formed only laevo; of 20 other strains, 13 formed dextro only and seven inactive only. He also stated that some strains of B.bulgaricus might produce small amounts of succinic acid, which might

account for its presence in cheddar cheese.

In a study of the lactic acid in cheddar cheese, Hart, Hastings, Flint, and Evans (1914) isolated organisms belonging to the group which they called Bacterium casei; one of these produced laevo lactic acid and two dextro. A mixture of the two types gave the racemic acid with a slight excess of laevo. They determined that two cultures held in sterile milk for several months produced among other acids much acetic, some propionic, but no formic, butyric, or caproic.

Hunter and Bushnell (1916) found that the average of the ratio of non-volatile to volatile acids produced by six cultures of B.bulgaricus from silage was 1.0 to 0.31; the acid was produced in milk incubated at 35° C. for 42 days.

Heinemann and Ecker (1916) determined the form of lactic acid produced in milk by three strains of the Boas-Oppler bacillus from gastric ulcers and found it to be laevo. This organism was considered identical with B.bulgaricus by Heinemann and Hefferan (1909) and by Galt and Iles (1915).

Orla-Jensen (1921) reported Thermobacterium bulgaricum (Bacillus bulgaricus) as producing up to 1.7% of laevo acid.

In a study of 15 cultures of Döderlein's vaginal bacillus and B.acidophilus, Jötten (1922) found that all formed inactive lactic acid; a slight amount of volatile acid was found.

McIntosh, James and Lazarus - Barlow (1924) reported that Dodds found B.acidophilus - odontolyticus, which some investigators consider as identical with L.acidophilus, produced malic acid with only a trace of lactic.

Pederson, Peterson, and Fred (1926) found that a sample of acidophilus milk contained a slight excess of laevo acid. The milk had not been sterilized before inoculation so the results are comparable to those found with naturally soured milks.

Kopeloff (1926) stated that unpublished results of Zoller show that "the non-volatile acid of acidophilus is found to be entirely dextro-lactic acid, no traces of succinic or other acid being present". Zoller further gave as his opinion that the stereomorphism of the lactic acid constituted one point of differentiation between L.acidophilus and L.bulgaricus. One culture of L.bulgaricus produced laevo lactic acid. He also reported that among the different strains studied uniformity was found

in the production of volatile acids, 5-10% of the total acid being volatile; these appeared to be about 50% formic, and nearly equal amounts of acetic and propionic.

Methods.

FORM OF LACTIC ACID: The method of determining the form of lactic acid by examination of the zinc lactates was followed in this study. The acid was produced by growing the organisms in flasks of milk held at 37° C. for one week. The lactates were then prepared by a dry or a wet extraction method.

DRY EXTRACTION METHOD: The whey was obtained from the fermented milk by heating the flask of milk in hot water and filtering off the whey through paper. The separation of the whey was usually facilitated also by the addition of 5 cc. of N/1 H_2SO_4 per 100 cc. of milk before heating. The whey was then evaporated down to a small volume over a water bath, after which plaster of Paris was added to the warm concentrated whey and rapidly worked in to prevent the formation of a firm mass. The proper amount of plaster of Paris readily took up all of the liquid and formed material which could be easily crumbled and handled. The lactic acid was extracted by putting the whey and plaster of Paris mixture in a thimble and extracting with ether for about 24 hours. The ether dripped on the

material in the thimble, seeped through it to the bottom of the container which rested on a hot plate, was evaporated and recondensed to complete the cycle. The ether and dissolved materials were transferred to a beaker and the ether allowed to evaporate, after which water was added and then $ZnCO_3$ in excess. The mixture was decolorized by boiling with animal charcoal. It was then filtered and the insoluble material washed on the filter paper with hot water. The filtrate was evaporated and allowed to crystallize as completely as possible because of the difference in the solubility of the salts of the active and inactive acids. The salts were recrystallized once and sometimes twice, dried and finely ground, after which they were allowed to air dry to practically constant weight.

WET EXTRACTION METHOD: The whey for the wet extraction method was obtained in the same manner as outlined above. The ether soluble material was then extracted from 600 cc. of the whey in a Kutscher and Steudel extraction apparatus, using an extraction period of 48 or 72 hours. The preparation, purification, and recovery of the zinc lactates was then done in the same manner as outlined for the dry extraction method.

The purified zinc lactates were studied in the

following manner: The percent H_2O of crystallization was determined by heating the air dry salts to practically constant weight at 108 to 110° C. With some of the preparations the effect of the salt on polarized light was determined, while with most of them the percent of ZnO was found by burning a known weight.

TOTAL AND VOLATILE ACID: For the determinations of the total and volatile acidities, 325 cc. portions of skim milk sterilized in pint milk bottles were inoculated with the organisms being studied and incubated at 37° C. for one week. The total acidity was determined by titrating 20 gram samples with N/10 NaOH using phenolphthalein as an indicator. The results were calculated in terms of percent lactic acid.

The volatile acid was determined by distillation as outlined by Hammer and Bailey (1919) and Hammer (1920). 250 gram portions of the fermented milk were distilled with steam after the addition of 15 cc. of approximately N/1 H_2SO_4. The first 1000 cc. of distillate were titrated with N/10 NaOH using phenolphthalein as an indicator and the results expressed as the number of cc. of N/10 NaOH required to neutralize the acid in this fraction of distillate. Although the method probably does not yield all of the possible acids it is considered suffic-

iently accurate for comparative results.

Results Obtained.

FORM OF LACTIC ACID: The data obtained from the study of the isomeric form of lactic acid produced in milk by 12 cultures of L.acidophilus are presented in Table X.

The percent H_2O of crystallization varied from 12.795 to 18.15, the lower figures apparently representing pure active salts (theoretical - 12.88% for active) and the higher figures pure or almost pure inactive salts (theoretical - 18.18% for inactive). Eight cultures gave acid which was largely active, and four gave largely, if not entirely, the inactive acid. Seven of the eight cultures giving active acid were from original isolations, while the eighth and the four giving largely inactive acid were from other laboratories. In every case in which the zinc lactate represented largely active acid the rotation was laevo so that the free acid was of the dextro form.

From the results of the study with these cultures of L.acidophilus it is evident that the type of lactic acid produced was not uniform but varied from pure active to practically pure inactive with mixtures of these be-

TABLE X.

Results on zinc lactates prepared from milk fermented by L. acidophilus cultures.

Culture	H_2O of crystallization			ZnO	Rotation
	det. A	det. B	ave.		
A1	12.85%	12.96%	12.905%	33.64%	l
A3	16.89	17.10	16.995	33.74	very slight l
A4	18.06	18.09	18.075	33.92	
A5	16.43	16.49	16.460	34.13	
A6	18.15		18.150		
S1	12.71	12.90	12.805	33.74	l
S5	12.88	12.71	12.795	33.50	l
S6	13.41	13.49	13.450	33.78	l
S7	13.78	13.50	13.640	34.50	l
S8c	14.04	14.10	14.070	33.78	l
S11	12.95	12.99	12.970	33.52	l
S12	14.34	14.28	14.310	35.49	l

tween the extremes.

The percent ZnO in the zinc lactates agreed quite well in most instances with the theoretical for this salt (theoretical - 33.46% for the anhydrous salt). In some instances the amount of salt available for this determination was rather small for highly accurate results.

There appears to be a certain amount of correlation between the type of acid formed by these cultures and some of the other characters which have been discussed. Six of the 12 cultures were classed as large and six as small (Table I). Four of these large strains grew at 45° C. but not at 15-20° C. (Table II). The same four were among six which failed to grow at a surface tension of 37.4 dynes in medium X although all grew at 40 dynes (Table V), and one of them did not ferment maltose (Table VII). These four were among five of the twelve which were secured from other laboratories and were also the same four which produced largely or entirely inactive lactic acid.

TOTAL AND VOLATILE ACID: Total and volatile acid determinations were made on 66 cultures; the data secured are presented in Table XI. The results on approximately half of the cultures represent duplicate determina-

TABLE XI.

Total and volatile acidities produced in milk during 7 days at 37° C.

Culture	Total acidity*	Volatile acidity	Culture	Total acidity	Volatile acidity
A1	1.34	29.8	C4	1.37	13.7
A3	2.02	34.5	C5	1.05	11.3
A4	1.37	33.8	C7	1.27	21.9
A5	1.59	33.9	C8	1.41	14.9
A6	1.17	21.4	C9	1.35	31.3
A7	2.03	35.8	C10	1.55	26.0
A8	1.77	41.7	C12	1.34	14.0
A9	1.76	16.7	C14	1.00	7.6
A10	1.57	14.8	C17	1.15	18.8
B1	1.35	26.6	C19	1.40	23.7
B2	2.24	23.5	C20	1.41	12.4
B3	1.74	6.9	C20a	1.58	7.7
B5	2.52	25.9	C21	1.42	23.8
B6	1.36	7.4	C22	1.38	11.5
B7	1.64	18.9	C22a	1.32	13.5
B8	2.48	27.8	C24	1.13	7.3
S1	1.26	27.8	C25	1.41	18.7
S5	1.35	19.4	C26	1.00	7.3
S6	1.07	33.0	C29	1.46	15.0
S7	1.40	26.0	C29a	0.69	6.5
S8a	0.52	5.7	C31	0.82	7.2
S8b	0.28	20.5	C32	1.33	14.5
S8c	1.98	31.8	C32a	0.87	7.3
S9a	0.56	4.7	C33	0.97	6.2
S9b	0.28	19.9	C36	1.21	32.3
S10	0.22	6.6	C40	1.46	8.5
S11	0.75	7.0	C41	1.50	12.3

A8	1.77	41.7	C12	1.34	14.0
A9	1.76	16.7	C14	1.00	7.6
A10	1.57	14.8	C17	1.15	18.8
B1	1.35	26.6	C19	1.40	23.7
B2	2.24	23.5	C20	1.41	12.4
B3	1.74	6.9	C20a	1.58	7.7
B5	2.52	25.9	C21	1.42	23.8
B6	1.36	7.4	C22	1.38	11.5
B7	1.64	18.9	C22a	1.32	13.5
B8	2.48	27.8	C24	1.13	7.3
S1	1.26	27.8	C25	1.41	18.7
S5	1.35	19.4	C26	1.00	7.3
S6	1.07	33.0	C29	1.46	15.0
S7	1.40	26.0	C29a	0.69	6.5
S8a	0.52	5.7	C31	0.82	7.2
S8b	0.28	20.5	C32	1.33	14.5
S8c	1.98	31.8	C32a	0.87	7.3
S9a	0.56	4.7	C33	0.97	6.2
S9b	0.28	19.9	C36	1.21	32.3
S10	0.22	6.6	C40	1.46	8.5
S11	0.75	7.0	C41	1.50	12.3
S12	1.59	25.5	C42	1.43	25.4
S15	1.46	18.0	C43	1.06	7.0
C1	2.09	21.1	C44	1.43	25.1
C2	1.42	28.9	C45	1.50	21.1
C3	1.78	6.9	C51	1.49	17.1
C3a	1.17	30.2	C52	1.32	31.8

* Calculated as percent lactic acid.

tions, while on the other half they represent single determinations, because such a close agreement was found between duplicates that single determinations were considered accurate enough for comparative purposes.

The total acid production varied quite widely, the lowest value found being 0.22% and the highest 2.52%. Ten cultures produced less than 1% acid, six more than 2%, and the remaining 50 between 1 and 2%. Among the ten lowest acid producers there seemed to be some correlation of characters: they were all classed as large; seven of them did not grow at 45° C., room temperature, or 15-20° C.; they were all among the cultures which grew slowly at 37° C., and three of them in over a year of frequent transfers never produced sufficient acid to cause coagulation of milk. Five of them seemed to be able to grow at a lower surface tension than the other five. Of the six cultures producing more than 2% acid three were in the L.bulgaricus group, two in the laboratory L.acidophilus group, and one in the L.casei group. All except the last were classed as large; grew readily at 45° C. but not at 15-20° C.; and failed to grow at the lower surface tensions.

The volatile acidities also varied widely, the

values found ranging from 4.7 to 41.7.* There did not appear to be any striking difference in the amount of volatile acid produced by the ten lowest acid producers except in two instances. Although the figures are low they are approximately proportional to the values obtained from the other cultures studied. The two exceptions noted deserve consideration inasmuch as both cultures were isolated from calf feces and exhibited almost identical characteristics throughout the study. These strains were among the three which did not produce sufficient acid to coagulate milk and yet the amount of volatile acid was higher in proportion to the total acid than in the case of any of the other cultures. Among the six high acid producers the two L.acidophilus cultures produced the highest volatile acidities although their total acid production was lowest among these cultures.

Considering the 66 cultures studied there does not seem to be any regular variations in the total or volatile acid production which can be considered of

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*As stated under methods these values represent the number of cc. of N/10 NaOH required to neutralize the acid in the first liter of distillate.

particular significance from the standpoint of identification and classification. It appears in a general way, however, that the L.acidophilus cultures produced slightly higher amounts of volatile acids than the others since values over 30 were obtained from several of them, none from the L.bulgaricus cultures, and from only an occasional L.casei culture.

PROTEIN DECOMPOSITION.

The role of L.casei in the ripening of cheese of the Emmental and Cheddar types has received a great deal of attention. Since the amount of soluble nitrogen in these types is known to increase during the period in which L.casei is dominant it is natural to assume that the breaking down of the protein is due to the activity of organisms of this species. Several investigators have shown this proteolytic action in milk. The proteolytic ability of L.acidophilus and L.bulgaricus has not occasioned as much investigation because this particular character has less economic significance than with L.casei. A comparison of the increases in the amounts of soluble nitrogen brought about by these organisms in milk seemed to be desirable in a study of their various important characters.

Review of Literature.

As early as 1897 von Freudenreich found that not only enzymes might be active in cheese but that the organisms of the B.casei type which were present in large numbers might be of considerable importance through their ability to increase the soluble nitrogen in milk. In 1899 he again took exception to the prevalent enzyme theory of cheese ripening by pointing out some of the chemical activities of the bacteria present in cheese during ripening. He reported Bacillus epsilon, which he considered the primary agent in the process, as producing considerable amounts of soluble nitrogen in milk cultures; the filtrate from a 9 months sample contained 0.235% N, and the filtrates from two 13 months samples 0.222% and 0.246% N. while the highest result from uninoculated milk was 0.033% N.

Orla-Jensen (1904) corroborated the work of von Freudenreich in a biological study of cheese ripening and presented data showing that Bacterium casei alpha and Bacterium casei epsilon were both vigorous destroyers of casein in milk to which $CaCO_3$ had been added, the latter being the more vigorous in this respect.

White and Avery (1910) stated that bacilli of the B.bulgaricus type had little or no action on casein

or fat in milk but that they produced a bitter acrid taste which might be due to the cleavage of these products.

Hastings, Evans, and Hart (1912) found that eight cultures of lactic bacilli after 3 months incubation in milk caused increases in the soluble nitrogen; the percentages varied from 12.5 to 62.5%.

Evans, Hastings, and Hart (1914) concluded that their Bacterium casei group was responsible for the pungent flavor developing late in the ripening period of both raw-milk and pasteurized-milk cheeses.

Hart, Hastings, Flint, and Evans (1914) reported that members of the B.casei group were able to form ammonia in milk but not in appreciable amounts.

Kulp and Rettger (1924) reported that L.bulgaricus and L.acidophilus broke down approximately 2 to 6% of the milk proteins; this was determined as the residual nitrogen after the peptone and di-amino nitrogen was found.

Weigmann (1924) reviewed the question of cheese ripening and brought out the fact that the B.casei types have been shown by numerous investigators to act on the casein, increasing the soluble nitrogen products such as amino acids and thus have an influence in pro-

ducing the characteristic flavor.

Methods.

The increase in soluble nitrogen brought about in milk, with and without $CaCO_3$, by some of the lactobacilli being studied was determined by the following method:

Four pint milk bottles containing 200 grams of skim milk were prepared for each culture: 8 grams of $CaCO_3$ and a few pieces of glass were added to each of two of the bottles. The total weight of each bottle and contents was recorded on a label placed on the cotton plug. After sterilization in the autoclave at 15# for 30 minutes and cooling, the four bottles were inoculated with the culture; this provided for duplicate determinations on each culture both with and without $CaCO_3$. They were incubated at 37° C. for one week after which they were held at room temperature for the remainder of a 30 day period in order to avoid excessive evaporation. The bottles containing the $CaCO_3$ were carefully shaken every day, the glass facilitating thorough distribution of the carbonate.

At the end of the incubation one cc. of glacial acetic acid was added to each bottle and the milk heated to 60° C. by standing the bottle in a pan of hot water.

The milk was held at this temperature for about 20 minutes and, after cooling, sufficient distilled water was added to correct the weight of each bottle to the original. The whey was then recovered by filtering through paper; the nitrogen content of the filtrates was determined by the Kjeldahl method, using 25 cc. portions. The distillate was collected in 15 cc. of N/5 H_2SO_4 and back titrated with N/10 NaOH with alizarin as an indicator. The soluble nitrogen in the uninoculated samples was determined in the same manner.

The total nitrogen of the original milk was determined by the Kjeldahl method. Calculated on the basis of 25 cc. of milk, the number of cc. of N/10 acid neutralized by the distillate was practically 100. The percent soluble nitrogen in the sterilized uninoculated and inoculated milk could accordingly be determined directly from the titration figures; the percent soluble nitrogen, then, was the figure obtained by subtracting the cc. of N/10 NaOH used in back titrating the 15 cc. of N/5 H_2SO_4 from the N/10 equivalent of this acid. The values from the duplicate uninoculated samples subtracted from figures similarly obtained on the inoculated portions gave the actual increase in soluble nitrogen due to bacterial action.

Results Obtained.

The results obtained on the proteolytic action on milk of 16 cultures of lactobacilli are shown by the data presented in Table XII.

Five L.acidophilus cultures from laboratory sources in milk without $CaCO_3$ caused increases in the percent of soluble nitrogen varying from 1.0 to 5.4%; four of these cultures in milk with $CaCO_3$ caused increases varying from 1.3 to 3.8% (the determination on the fifth was not made). The culture causing the highest increase in the milk without $CaCO_3$ did not cause the highest increase with $CaCO_3$. Two isolated L.acidophilus cultures in milk without $CaCO_3$ caused increases of 1.6 and 4.7%; in milk with $CaCO_3$ the increases were 2.5 and 11.8% respectively.

Four L.bulgaricus cultures in milk without $CaCO_3$ produced increases of 1.0 to 6.7%; with $CaCO_3$ the increases were 3.2 to 19.2%, the highest results in each case being with the same culture. The increases were higher in the milk with $CaCO_3$ in every instance.

Five L.casei cultures in milk without $CaCO_3$ caused increases of 1.2 to 3.5%; with $CaCO_3$ the increases were 2.6 to 14.0%, the highest results in each case being with the same culture. The increases with this group of

TABLE XII

Data on increase in soluble nitrogen in milk inoculated with various cultures.

Culture	Percent increase	
	without $CaCO_3$	with $CaCO_3$
A1	1.0	3.8
A3	5.4	2.9
A5	4.4	3.4
A7	4.0	
A8	2.3	1.3
B1	1.0	3.2
B2	3.3	11.8
B3	3.7	8.4
B5	6.7	19.2
C3	3.5	14.0
C12	1.2	2.6
C23	1.7	5.6
C29	1.8	5.7
C32	1.9	4.5
S8c	1.6	2.5
S12	4.7	11.8

cultures were also higher in the milk with $CaCO_3$ in every instance.

Among the 16 cultures, the eight classed as large caused greater increases in the soluble nitrogen of milk without $CaCO_3$ than were caused by any of six classed as small. The other two small cultures gave results somewhat higher than most of the large cultures. The same relationship did not exist among the cultures in the milk with $CaCO_3$. Four L.casei cultures classed as small caused increases greater than were caused by four L. acidophilus cultures classed as large. One of these L.casei cultures gave the second highest value among the 15 determined. The two L.acidophilus cultures classed as small caused increases greater than were caused by four cultures classed as large which were also L.acidophilus. The other two cultures classed as small, one an L.bulgaricus and one an L.casei, caused increases which were very slightly higher than two or three of the cultures classed as large but lower than ten of the 15 cultures.

Attention should be called to the fact that all of the cultures were able to increase the amount of soluble nitrogen in milk to some extent although in some cases the increase was slight.

Discussion of Results.

There appears to be a certain amount of correlation among several important characters of the lactobacilli included in this study. The correlation is particularly striking between the size of the organisms, the temperatures of growth, the influence of the surface tension of the medium on growth, and the character of the acid produced in milk. No very significant relationships were found in the ability of the various types to attack proteins in milk.

Among the organisms in both L.acidophilus groups and the L.bulgaricus group, practically the only ones which grew at 45° C. were classed as large. In the L.casei group, however, only one of the 11 which grew at 45° C. was large. More of the total number of cultures were able to grow at room temperature than at 45° C. or 15-20° C. At 15-20° C. practically all of the cultures which grew were classed as small. This fact was particularly significant in the group of L.acidophilus from laboratory sources, in the L.bulgaricus group, and in the isolated L.acidophilus group because it evidently was also correlated with some of the other outstanding characters and suggested the possibility that some of the large types considered as L.acidophilus should be con-

sidered as more closely related to some other group. It was quite evident that these same large cultures were inhibited by low surface tensions in the media used. Most of them were inhibited when the surface tension was reduced very much under 40 dynes, while the other cultures were able to grow at nearly 37 dynes in the same media. Similarities were also found between several of the laboratory L.acidophilus and the large L.bulgaricus cultures in that nearly all of them were inhibited at a surface tension of 39 dynes when maltose was substituted for lactose in medium X. One of these L.acidophilus cultures and three L.bulgaricus failed to grow in the medium containing maltose without depressant. The small cultures in these groups apparently grew as well with maltose as with lactose in the medium. In general the L.casei cultures seemed able to grow at nearly as low surface tensions as the L.acidophilus types; most of the variations occurred with the cultures which were naturally slow in growth.

In the study of the type of lactic acid formed by 12 cultures considered as L.acidophilus there also appeared to be some correlation between the characters just discussed and the type of acid. Six of the cultures were large and six small. The type of acid varied from

practically pure active to practically pure inactive; the active acid was always the dextro modification. Four large strains produced largely inactive acid; these cultures grew at 45° C. but not at 15-20° C.; they failed to grow at 37.4 dynes in medium X although all grew at 40 dynes; one of them failed to ferment maltose. These four were among five of the twelve which were secured from other laboratories as representative L.acidophilus strains.

Determinations were made on the increase in the percent of soluble nitrogen produced in milk by 16 cultures from the four groups of organisms. The increases in the milk without $CaCO_3$ varied from 1.0 to 6.7% and in the milk with $CaCO_3$ from 1.3 to 19.2%. No striking correlation seemed to exist between the various characters of the organisms and the extent to which they were able to decompose the milk proteins. Each culture studied, however, was able to cause an increase in the soluble nitrogen in milk both with and without $CaCO_3$.

PART III.

FEEDING EXPERIMENTS.

Introduction.

No other point with reference to the lactobacilli has been so extensively studied and discussed as that of the value of certain of these organisms in combating the effects of intestinal bacteria whose activities are deleterious to the health of the individual harboring them. Volumes of literature have been devoted to considerations of the pros and cons of the subject and undoubtedly numerous problems remain to be solved.

Although not the first to suggest the use of bacteria in a therapeutic role, Metchnikoff (1907) through his observations and conclusions regarding the causes and remedies for early senility first brought the possibilities of such a method for alleviating intestinal disorders before the public. His ideas immediately gained credence over a wide area and the use of so-called Bulgarian buttermilk became a common remedial measure for intestinal disorders of various kinds.

It was not many years, however, before doubts began to appear as to the ability of the organism of Bulgarian buttermilk, L.bulgaricus, to survive conditions in the body and become established in the intestinal tract where

it was supposed to inhibit the activities of the putrefactive types by its acid production. Many other theories were advanced in an attempt to show that beneficial results were being obtained from other agencies, such as the lactic acid in the fermented milk, rather than from the actual presence of the L.bulgaricus. Finally it was proposed that L.acidophilus and not L.bulgaricus was the logical organism to use for such a purpose and the idea was advanced that it was capable of permanent implantation and that L.bulgaricus was not.

The pioneer work of Fisher (1919) in feeding cultures of L.acidophilus led the way for a great number of experiments on the effects of feeding L.acidophilus and L.bulgaricus cultures to humans and animals. The effect of introducing these organisms into the intestinal tract on the production of toxic substances by other organisms, and on the functioning of the intestinal tract in eliminating waste material, and the everpresent question of how to differentiate between the two species are points which have been abundantly discussed.

It was not considered within the scope of the present work to conduct a great deal of experimental feeding but certain of the outstanding characters of L.casei cultures were so similar to those of L.acidophilus

cultures that it suggested that if one was capable of being implanted in the intestinal tract the other might also. Information on this particular point is also desirable from the standpoint of the dairy industry because of the prevalence of the L.casei organisms in certain types of cheese and their presence in milk. Liberal consumption of such products then should assure the introduction of this type into the intestinal tract through natural channels of the diet. For these reasons part of the work was devoted to feeding experiments with humans and animals.

Review of Literature.

Quite a few years before Metchnikoff advanced his theories on the value of fermented milk in combating intestinal disorders, Herter (1897) had concluded that the introduction of large numbers of lactic acid bacilli into the intestines might markedly reduce the indican and ethereal sulfates of the urine. He likewise stressed the value of carbohydrates and a bread and milk diet in decreasing the amount of products of putrefaction in the urine. These observations confirmed the work of a long series of investigations of others which had begun as early as 1868 with studies on the toxicity produced by protein decomposition in the intestines. Numerous

investigations following the work of Herter have been reported showing the value of certain carbohydrates in inhibiting intestinal putrefaction.

Poehl (1887), Rovighi (1892), Tissier and Martelly (1902), Tissier and Gasching (1903) and others reported the favorable effect of sour milk and lactic acid in decreasing intestinal putrefaction.

Working under the direction of Metchnikoff, Cohendy (1906a,b,c.) published several papers giving the results of experiments which indicated the successful implantation of B.bulgaricus in the intestinal tract of humans. Combe (1907) suggested the value of the organisms in yoghurt for overcoming putrefactive types, and Belonovsky (1907) in experiments with white mice concluded that B.bulgaricus was implanted in the intestines and reduced the putrefactive types rapidly. Oehler (1911) fed yoghurt to mice and monkeys and found that the yoghurt bacilli could easily be demonstrated in the feces during the feeding but that they disappeared rapidly when the feeding was stopped so that no implantation could have taken place.

Other reports, contrary to the theories of Metchnikoff, were published by Luerssen and Kühn (1908), Herter and Kendall (1908), Kendall (1910a), Heinemann

(1912), Distaso and Schiller (1914) and others.

That diet has a marked influence on the intestinal flora and that carbohydrates and milk bring about a favorable change to the desirable aciduric type both in humans and various animals has also been reported by numerous investigators. Prominent among these are the work of Herter and Kendall (1908 and 1910), Torrey (1915 and 1919), Rettger and Horton (1914), Hull and Rettger (1914 and 1917), Rettger, Kirkpatrick, Jones (1914); Rettger, Kirkpatrick, and Card (1915), Rettger (1915), Cannon (1921), Kopeloff and his co-workers (1923-1927), and others.

With regard to the more recent work on the influence of diet on the excretion of indican and the phenols Underhill and Simpson (1920) found that the excretion of these substances varied with the protein intake but that a large amount of lactose lowered the amount excreted. Smith and Kulp (1922) reported that the favorable results of B.acidophilus cultures in gastro-intestinal cases did not depend primarily on a decreased production of indican and ethereal sulfates. Kast, Short, and Croll (1922) stated that if indican might be taken as an index of intestinal putrefaction it appeared from their results that implantation of B.acidophilus in the

intestines did not necessarily lower putrefactive processes.

Excellent reviews of the literature dealing with the subject of intestinal bacteriology and the effect of diet on the types of bacteria and intestinal condition have been presented at various intervals. Among these, reference should be made especially to Herter (1907), Kendall (1911), Logan (1914), Bushnell and Frey (1917), Rettger and Cheplin (1921) and Kopeloff (1926).

EXPERIMENTS WITH HUMANS.

Methods.

The general plan of the feeding experiments with humans was to select persons who would consume a quart of fermented milk each day and then to make a bacteriological study of the feces at as frequent intervals as the conditions permitted. The four subjects reported on were all young men in normal health. The milk was consumed at any time during the day suiting the convenience of the subjects and the customary mixed diet was not otherwise changed.

The fermented milk was prepared in Erlenmeyer flasks in the same manner that acidophilus milk is ordin-

arily prepared in the laboratory. The cultures were carried individually and were mixed only at the time of inoculating the flask of milk with an approximately equal amount of inoculum from each culture.

The feces were collected in waxed paper containers and the examinations made usually within a very short time although in some instances not until after two or three hours. A method having features of those used by Rettger and Cheplin (1921) and by Tsuchiya (1926) was employed. A suspension of the feces was made in 5 cc. of sterile physiological salt solution to match tubes nine to eleven of the McFarland (1907) nephelometer scale. From these suspensions a loopful was spread on a glass slide over an area of one square centimeter, fixed and stained by the Gram method for microscopic examination. Two plates were poured with whey agar, using one loopful of the standard suspension in one tube of the agar and three loopsful from the first into the second. This was found to give a satisfactory distribution for mass study and for single colony isolation. Two other tubes of whey agar were inoculated in the same manner and mixed thoroughly by tilting back and forth, after which they were allowed to solidify. A third inoculation of one loopful of the suspension was made

into a tube of litmus milk. The plates and tubes were incubated at 37° C. for 48 hours and then examined.

Frequent isolations were made by picking colonies from the plates into litmus milk and the cultures subsequently examined microscopically and culturally with reference to some of the characters discussed in a previous part of the work.

Results Obtained.

CASE-1.

The fermented milk used in this case contained five L.casei cultures - C10, C12, C17, C19, and C28.

Two fecal specimens were examined before the first fermented milk was taken. The direct smear of the first showed Gram-negative rods strongly predominant with only a few large Gram-positive rods and cocci. Very few small colonies were found on the whey agar plates and none of the woolly acidophilus type. The most numerous colonies were of a large irregular flat type. The smear of the second preliminary specimen contained a fairly large proportion of Gram-positive rods, many of which were of the slender type characteristic of L.acidophilus. Approximately 10 - 15% of the colonies on the plates were rough, irregular, small and the rest the large irregular type.

The first specimen examined after the consumption of the fermented milk began was on the second day. The fecal smear showed about 10-15% Gram-positive organisms, among which were a noticeable number of small slender rods. Approximately 80% of the colonies on the plates were of the small irregular type characteristic of the L.casei cultures being used. On the third day the smear presented practically the same picture, and the plates a slightly larger proportion of the small irregular type of colony.

During the remainder of the ten day test period the proportion of Gram-positive rods in the fecal smears increased to approximately 40% on the eighth day and this was the highest proportion reached. By the third and fourth days the proportion of small irregular colonies on the plates had reached approximately 95%, and this number was maintained almost constantly during the test period. From the second day on numerous colonies were picked into litmus milk. From the thickly seeded plates a mass inoculation of several of the small colonies was made and in practically every instance the milk coagulated promptly and stained smears showed the organisms to be the typical L.casei rods. A very large proportion of

single colonies picked from the less heavily seeded plates gave the same results, showing quite conclusively that the organisms taken in the fermented milk were being recovered.

No very significant results were obtained from the direct inoculation of the fecal suspension into litmus milk or from the whey agar shakes, since in this case practically no gas was produced in either even before consumption of the milk began. The type of colonies in the shakes did change somewhat, however, from a large smooth type near the surface to a very small type which appeared principally in the lower half of the agar.

Observations on the stools were made after the milk was discontinued at intervals of a day or two for eight days, again in one week, and the last time after two more weeks. During the first week there was a slight change in the number of Gram-positive rods, the proportion falling to 25-30%. The proportion of typical L.casei colonies on the plates remained almost the same. At the end of the second week the smear was practically the same as at the end of the previous week, but the proportion of small irregular colonies on the plates had fallen to 45-50%. At the end of the fourth week the number of Gram-

positive rods had fallen to 15%, and the small irregular colonies to 40-45%.

Within a day or two after the use of the fermented milk was begun a change occurred in the character of the feces and this continued for several days after the milk was dropped from the diet. The color of the feces became much lighter; the consistency changed from hard and dry to soft; and the odor was less disagreeable. In this case also the number of regular daily defecations was increased and a tendency toward constipation noticeably alleviated.

CASE-2.

The fermented milk used in this case contained the same L.casei cultures that were used in the previous case.

The subject in this case habitually used a quart of milk every day with his meals. The examination of three preliminary fecal specimens showed that about 50% of the organisms were Gram-positive with slender rods predominating among them. The plates contained from 90 to 95% small woolly colonies typical of L.acidophilus. On inoculation into litmus milk the organisms did not cause coagulation within seven days at least, although they were slender Gram-positive rods.

On the second day after consumption of the fermented

milk was begun the smears contained 50-60% Gram-positive rods, practically all of which were small and slender. The colonies on the plates were about 50% of the small woolly type and 50% of the small irregular type formed by the cultures being fed. On the fourth day the Gram-positive rods appeared to be about 50-60% of the total and the small irregular colonies on the plates had reached a proportion of about 95%; both remained practically the same during the rest of the two weeks feeding period. The small woolly colonies disappeared from the plates almost entirely. When these conditions had apparently become established numerous cultures were isolated from single colonies and by mass inoculations; practically all showed characteristics of the L.casei types. Litmus milk was rapidly reduced and coagulated, the cultures grew readily at room temperature and most of them very poorly or not at all at 45° C.; they grew quite well in whey peptone broth at 39.0 dynes.

A slight amount of gas was evident in the litmus milk tubes from the preliminary specimens and a day or two after consumption of the fermented milk began, but during the rest of the trial most of them showed no gas or only a very slight amount. Stained smears showed nearly all the organisms in the milk to be slender Gram-

positive rods. Practically no gas was obtained in any of the agar shakes. After a day or two of consumption of the fermented milk from a half to two thirds of the colonies were very small and located in the lower two thirds of the agar.

In one specimen taken a week after the fermented milk was discontinued there was a slight reduction in the number of Gram-positive rods in the smears and the colonies on the plates showed reversion to about 80-90% of the small woolly type.

It was realized after examining the preliminary specimens that the case was extraordinary in the number of Gram-positive rods already present but the complete change in the type of colonies on the plates and the cultural tests seemed to indicate transformation of the flora to the type being consumed in the milk culture. Reversion to the type present at the beginning apparently took place rapidly when the cultured milk was discontinued.

The character of the feces showed very little change because their condition at the beginning was already typical of that usually brought about by the use of milk in sufficient amounts to establish the aciduric flora.

CASE-3.

The fermented milk used in this case contained the same L.casei cultures that were used in the previous cases.

Two preliminary specimens were examined before the consumption of the fermented milk began. The smears contained almost exclusively Gram-negative organisms. The very few Gram-positive forms were large thick rods, cocci, and nearly oval cells. The plates from the first specimen contained about 45-50% large spreading woolly colonies; the rest were large smooth colonies which smears showed to be Gram-negative organisms of the colon type. Woolly colonies picked into litmus milk produced no change in ten days to two weeks. On the plates from the second specimen almost no woolly colonies were present; the large smooth colonies were practically the only type.

On the first day after consumption of the fermented milk was begun the smear showed a few slender Gram-positive rods, although the appearance of the colonies on the plates had not changed. The number of Gram-positive rods then increased quite rapidly to 35-40% on the third day and remained fairly constant until the twelfth day when it had increased to about 45%. On the second day about 15-20% of the small irregular colonies appeared on

the plates. This proportion increased rapidly in the next day or two to the same dominant percentage found in the previous cases - practically 95%, where it remained fairly constant during the rest of the two weeks period. Cultures isolated from the plates when the flora had evidently been changed showed the general characteristics of the L.casei types, essentially as described for the cultures in the previous case except that a larger number were able to grow at 45° C.

Practically no change occurred in the appearance of the whey agar shakes other than an increase in the proportion of small colonies deep in the agar and diminution in the number of large colonies near the top. The litmus milk tubes did show more change, however, than in the previous cases. The fermentation from the preliminary specimens was very stormy and there was a considerable amount of whey and a disagreeable odor. From about the third day of the test period the character of the fermentation was very different. For five or six days there was no gas, very slight whey, and a good odor. Numerous smears also showed the presence of large proportions of the small slender Gram-positive rods. Then for several days there was a return of a slight gassy condition although there was still no gas in the agar shakes.

One specimen nine days after the milk was discontinued still showed about 20% Gram-positive organisms in the smear, a fairly large proportion of which were slender rods. About 50-60% of the colonies on the plates were of the small irregular form, and some of the woolly type were also present. There seemed to be no change in the appearance of the litmus milk or the agar shakes.

The character of the feces changed quite rapidly after consumption of the fermented milk was begun. The color became pronouncedly lighter and the odor less disagreeable. There was not much change in the consistency because the stools were at no time hard or dry.

CASE-4.

The fermented milk used in this case contained L.casei cultures C12, C17, C19, and C28. Culture C10 was omitted because it was the only one among the five which was able to grow at 45° C. and it seemed desirable to see if different results would be obtained without it in the mixture.

The smear from the one preliminary specimen contained about 50% Gram-positive organisms which were practically all short thick rods and cocci. The plates contained 80 - 85% small smooth colonies which were shown to be Gram-negative. None of the woolly or rough edged

types were found. There was not much change in the total number of Gram-positive organisms during the twelve days that the cultured milk was consumed but the small slender rods began to appear noticeably by the second and third days and then increased to approximately 40% of the Gram-positive organisms by the eighth day. The type of colonies on the plates did not change as completely as in the previous cases to the small irregular type, although by the fifth and sixth days the small woolly and irregular types together had reached the proportion of 85 - 90%. During the remainder of the test period the woolly type was noticed around the large smooth colonies, while away from these only the irregular colonies were found. In point of total numbers the small irregular colonies strongly predominated. The cultures isolated from the irregular colonies exhibited the same characteristics as those in the previous cases, while those from the woolly colonies appeared to be principally large irregular shaped Gram-positive rods which grew very slowly in milk at 37° C.

The appearance of the whey agar shakes changed from a slight gassy condition to no gas and a large proportion of the small colonies deep in the agar. The litmus milk fermentation was stormy until three or four days after

the consumption of the cultured milk began after which the gas was noticeably diminished. By the seventh day practically no gas was formed.

One specimen taken four days after the milk was discontinued showed no change from that which existed at the end of the test period.

A greater change in the character of the feces was evident in this case than in any of the previous ones. From a dark brown color, a hard, dry consistency, and a foul odor they gradually became lighter, softer, and less disagreeable in odor. The change was very pronounced by the seventh day of the test period.

EXPERIMENTS WITH RATS.

Methods.

The general plan of the experiments with rats was to feed a diet supposedly conducive to the growth of a proteolytic flora and then add to this diet pure cultures of the organisms it was desired to study and observe the effects by bacteriological examination of the feces.

Young healthy rats were placed in individual cages over a quarter inch mesh wire screen for the entire duration of the feeding period. They were then fed the basal diet until the intestinal flora, as shown by stained smears, had become predominantly Gram-negative. The

usual length of this preliminary feeding was from six to ten days. The basal diet in the principal set of experiments consisted of 10 grams of fresh white bread and 3 grams of fresh, lean chopped beef.*

The organisms for feeding were grown on whey agar slants prepared in 7 x 1 inch test tubes inoculated from fresh milk cultures and incubated at 37° C. for 48 hours. Mixtures of several cultures were not used; each rat received a single strain. In part of the work the growth was removed by means of a large platinum wire flattened and bent at the end to form a scraper, while when lactose was added to the diet the growth was removed by washing. In either case the bacteria were suspended in two cc. of sterile physiological salt solution until the turbidity matched tubes five to six of the McFarland nephelometer scale. The suspensions, which were used within a very few minutes in order to avoid possible injury to the organisms, were added to the diet by pouring over the mixed bread and meat at the daily feeding.

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* In preliminary feeding trials the basal diet was made up of white bread and meat both of which were dried and ground. It was found, however, that the rats did not consume it readily. Some of them left a great deal of the meat while others left bread so that the results from different rats were not considered comparable. The fresh bread and meat diet was consumed with very little waste.

The feces were obtained directly in a test tube containing 5 cc. of sterile saline solution by holding the rat up by the tail and if necessary rubbing the back at the base of the tail. During the early part of the work pieces of glass were used in the test tubes to help in breaking up the feces, but it was later found that this could be done more quickly and easily by crushing the feces in the solution with small sterile glass rods. The suspensions were made to match as closely as possible tube 8 of the McFarland nephelometer scale.

The fecal examinations were made in the manner described under the methods used in the human experiments.

Results Obtained.

The standard bacterial suspensions were added to the diet after six days of feeding the basal diet alone. At that time the fecal smears showed the flora to be predominantly Gram-nagative. The proportion of Gram-positive organisms varied from about 5% to 15 or 20% and in practically every case these were large thick rods, cocci, and oval forms. Cultures A1, S6, C7, and C17 were each fed to one rat and three rats were fed the basal diet to serve as controls. After one week one gram of lactose was added to the diet of each rat receiving a bacterial suspension and was continued to the end of the feeding period.

The proportion of Gram-positive organisms showed very little change, the highest number being about 25% in the feces of the rat being fed culture C7; the character of the organisms in the smears from the control animals was quite different, however, from those of the rats receiving cultures. The presence of small slender rods was much more noticeable with the latter, while the controls showed practically all large rods, or cocci. In order to eliminate errors introduced by knowing the source of each smear, an unbiased, experienced bacteriologist examined and described the smears from the last three days of the experiment. The rats at that time had been receiving the bacterial suspensions 23 days and in addition one gram of lactose each day for the last 16 days. In only one instance was a smear from a control rat picked out as one from a rat receiving a culture. The smears from the control animals were in all other instances described as containing Gram-positive cocci or large thick rods, but very rarely or not at all any small slender rods. The other smears were described as containing many slender Gram-positive rods. As previously indicated, however, the Gram-positive organisms never reached a predominating proportion.

The number of small irregular and woolly colonies

on the plates when the cultures were first fed varied from about 10 or 15% to 85 or 90%. Within a few days after the cultures were added to the diet the colonies became predominantly the small irregular type with few woolly ones, although much greater daily variations were observed than in the experiments with humans. Cultures isolated from these small irregular colonies, many of them by mass inoculation, were largely small slender Gram-positive rods which showed the general characteristics of the cultures being fed. With the control animals, on the other hand, the colonies were predominantly of the small woolly type, and these were found to be large Gram-positive rods which grew very poorly in milk. The results with the control animals were particularly confusing since a predominantly Gram-negative flora and the character of the Gram-positive organisms in the smears apparently indicated that the smooth colonies of Gram-negative types might be expected on the plates.

There was very little if any difference between the whey agar shakes from the animals being fed cultures and the controls. In all cases there was very little gas and in general the number of small and large colonies seemed to be about the same. The litmus milk fermentations were much more stormy than was the case in the ex-

periments with humans. Although almost no tubes were obtained free from gas the amount seemed to be somewhat less throughout the feeding period with the rats receiving cultures than with the controls.

There was no noticeable difference in the character of the feces either in individual animals or between the test animals and the controls except that upon the addition of lactose softer and lighter feces occasionally appeared.

Discussion of Results.

When four human adults consumed daily one quart of milk fermented with a mixture of several L.casei cultures, the changes which took place in the appearance of fecal smears, in the type of colonies on plates from fecal suspensions, and in the type of fermentation produced in milk inoculated with fecal material all seemed to indicate at least partial transformation of the intestinal flora. The longest period that stools were examined after the fermented milk was discontinued was four weeks; in that case the L.casei type of rods were still apparently present in considerable numbers. In the other three cases the length of time that the stools were examined after discontinuing the milk was too short to determine as satisfactorily the persistence of the L.casei types in the intestines.

The important facts which seemed to be indicated by the results of these four trials were that L.casei strains isolated from milk and cheese had characters which made their implantation in the intestinal tract possible. These types of lactobacilli have many characters in common with the L.acidophilus types particularly in respect to temperatures of growth and ability to grow under reduced surface tensions. The latter character particularly has been suggested as a possible explanation for the ability of L.acidophilus to become implanted, and as a reason why L.bulgaricus can not. The natural occurrence of L.casei in milk and cheese offers a possible explanation for the presence of organisms of this type in the feces of persons consuming large amounts of these products. Many of the organisms which have previously been called L.acidophilus should perhaps have been classed as L.casei.

The value of these facts to the dairy industry is evident. The presence of these organisms in dairy products may be an additional factor in the value of these products as a food. If greater concentrations of the organisms are desired, they may be readily isolated from milk or cheese and pure cultures prepared as in these experiments. The value of such fermented milk preparations in treating abnormal conditions in the intestinal

tract was not determined since only normal cases were observed.

Although the results secured from feeding pure cultures of L.acidophilus and L.casei to rats were not as definite as those secured from the experiments with humans they did indicate that implantation of the L.casei cultures was as successful as implantation of L.acidophilus cultures under the same conditions. The difficulty encountered in this series of experiments seemed to be in establishing abnormal conditions in the rats so that greater differences between the control and test animals might have been produced.

PART IV.

PREPARATION OF ACIDOPHILUS MILK AND THE EFFECT OF VARIOUS TEMPERATURES ON THE VIABILITY OF L.ACIDOPHILUS.

Introduction.

A study of a group of organisms which have created so much popular interest within the last few years seemed to demand that a certain amount of attention be given to phases of the problem which are of commercial significance. This part of the work includes a study of different methods of preparing

acidophilus milk which would be suitable for commercial conditions, and a study of the effect of various temperatures of storage on the viability of L.acidophilus in milk cultures intended for therapeutic purposes.

Review of Literature.

Rettger and Cheplin (1921) in describing the preparation of acidophilus milk reported the usual method of autoclaving the milk at 15# pressure for 20 to 30 minutes before inoculation and another procedure in which the milk was heated in a container submerged in boiling water for 45 minutes to one hour. Milk heated in the latter way, inoculated and held at 30°C. and 37°C. underwent characteristic acidophilus coagulation. The uninoculated flasks at these temperatures showed decided evidences of putrefaction within 24 hours. At 20° C. similar but much slower changes took place. They concluded that, from the practical standpoint, sterilization would be necessary to obtain constant and reliable results.

Kopeloff and Cheney (1922) found holding of acidophilus milk for three or four days, at room temperature, to be satisfactory; after this the viable organisms decreased rapidly and the acidity increased to the point of unpalatability.

Without the presentation of data Bass (1924) stated

that acidophilus milk prepared from milk heated to 190-195° F. for one hour, held 3-4 hours at 98° F. and then reheated at the same exposure was good for four or five days, after which the number of viable bacilli diminished rapidly; the keeping quality was not improved by refrigeration.

Kopeloff (1926) presented data on three trials in which acidophilus milk was held at ice box temperature (9° C.); the averages showed that after three days about 90% and after five or six days 97-99% of the original number were not viable. In an effort to kill the organisms for another experiment some acidophilus milk was held at -3° C. for one week, remaining frozen except when thawed enough to remove samples. The original count of about 50,000,000 per cc. was reduced to 1,000,000 per cc. in two days, 500,000 per cc. in four days, and 25,000 per cc. in six days, or a reduction of 99.95%.

Mortensen and Gordon (1911) in a bacteriological study of lacto found that the lactic acid bacteria survived freezing for a long period and that other forms present did not survive. Their curve, plotted from the averages of 8 trials, showed that of approximately 2,000,000,000 organisms at the beginning, 600,000,000 survived after 7 days, 300,500,000 after 14 days, and

200,000,000 after 21 days.

COMPARISON OF METHODS OF PREPARATION OF ACIDOPHILUS MILK.

The most satisfactory method of preparing acidophilus milk is a question of considerable commercial importance. The usual laboratory method of preparation is to sterilize the milk before inoculation, but such a practise is difficult, if not impossible, in most commercial plants without a considerable outlay for equipment. Sterilized milk also has a brown color and a characteristic caramel flavor distasteful to many persons so that a white product without the caramel flavor is highly desirable. It has become necessary then in many cases to rely upon double pasteurization with incubation between the heating periods during which the resistant organisms presumably enter a vegetative state and are then destroyed by the second heating. The important question is in regard to the purity and general commercial suitability of such a product.

Methods.

Acidophilus milk has been prepared for some time by the Iowa State College Dairy Department for its customers. This milk has been fermented in flasks and in

bulk; consistently good results have been secured although an occasional off flask or large batch has been obtained. The bulk milk has been prepared in a jacketed glass lined steel tank of about twenty gallons capacity. The tank is provided with a propellor agitator and a heavy lid which is bolted down to provide an air tight container in which the milk is raised conveniently to a temperature of 220° F. with a steam pressure of about 6# in the jacket, thus providing a satisfactory means for sterilizing milk in bulk. The milk is inoculated with a flask of acidophilus milk without opening the top by removing the safety valve in the lid and pouring the inoculum through the opening. It is then held at 98-100° F. for the required incubation period, when it is cooled by circulating cold water through the jacket; it is drawn off through a valve in the center of the bottom.

The efficiency of the double pasteurization method for preparing acidophilus milk was studied both with small amounts in flasks and with large amounts in practical dairy equipment. The milk in small amounts was heated by placing two flasks in a pan of hot water on a gas heater; one of the flasks held a thermometer in the cotton plug so that the temperature could be controlled within one or two degrees. One flask of milk was inoculated, one

held uninoculated, and a third from the same milk sterilized and inoculated in the usual manner for comparison.

The study of the preparation of milk in bulk was carried out in a 30 gallon Haughdahl starter can, and in the glass lined steel tank described above.

Results Obtained.

The results obtained in four trials on preparing white acidophilus milk in two-liter flasks are shown by the data presented in Table XIII.

In the first trial both the uninoculated and the inoculated milk had a very bad flavor and odor; it would have been absolutely unsalable. In the last three of the four trials the resulting product was satisfactory as far as palatability was concerned, although a few large thick Gram-positive rods were present as a contamination. Other than the presence of these rods there was nothing about the milk which would have prevented its use as a satisfactory commercial product. The uninoculated milk, treated in exactly the same manner as the inoculated, coagulated in these three trials within two or three days. As would be expected, in each case the odor and flavor were bad. The acidity was not high enough to cause coagulation so the effect was one typical of the sweet curdling spore forming organisms common in milk. In every

TABLE XIII.

Data on preparation of "white" acidophilus milk in small amounts.

Trial no.	Treatment	Quality of resulting product.	
		uninoculated control	inoculated
1	185 - 189° F. 1 hour. 3 hrs. at 37° C. 185 - 190° F. 1 hour.	bad odor, curd shrunken, wheyed off, bitter flavor, many large Gm.+ rods.	bad odor, curd shrunken, wheyed off, bitter flavor, many large Gm.+ rods.
2	195° F. 1 hour 3 hrs. at 37° C. 195° F. 1 hour	coagulated in 2 days, bad odor, many large Gm.+ rods.	good flavor and odor, an occasional large Gm.+ rod.
3	200° F. 1 hour. 3 hrs. at 37° C. 200° F. 1 hour.	coagulated in 3 days, bad odor, many long twisted Gm.+ rods in filaments.	good flavor and odor, an occasional large Gm.+ rod.
4	200° F. 1 hour. 3 hrs. at 37° C. 200° F. 1 hour	coagulated in 2 days, very bad odor, long filaments and thick Gm.+ rods.	fair flavor and odor, a few large Gm.+ rods.

trial except the fourth the milk prepared by the usual sterilization method was entirely satisfactory. In trial four the milk was of such poor quality that it coagulated during sterilization, but in spite of that a fair product was obtained by the double pasteurization method.

It appears from the results of these trials that in order to produce a satisfactory white product it is advisable to use exposures of about 195 - 200° F. for one hour with an incubation period of about three hours between heatings. The milk should be of good quality to assure obtaining a finished product with a satisfactory flavor and as few undesirable organisms as possible.

The results of seven trials in which larger quantities of milk were treated under different conditions are presented in Table XIV. No uninoculated control milk was held but milk sterilized and inoculated in flasks during the course of these trials produced the usual satisfactory acidophilus milk.

Two trials using the Haughdahl starter can and two using the glass lined tank for treating the milk resulted in unsatisfactory products. The milk had a bad flavor and odor and was heavily contaminated with the usual large Gram-positive rods. As shown by the data the

TABLE XIV

Data on preparation of "white" acidophilus in bulk.

Trial no.	Container	Treatment	Quality of resulting product.
1	Haughdahl starter can	190 - 194° F. 50 minutes	wheyed off, lumpy curd, bad odor and flavor, large Gm.+ rods.
2	Haughdahl starter can	185 - 195° F. 50 min. 4 hrs. at 100° F. 185 - 195° F. 50 min.	ropy, bad odor and flavor, short thick Gm.+ rods.
3	Glass lined tank	190° F. 30 min. 3 hrs. at 100° F. 190° F. 30 min.	wheyed off, bad odor and flavor, large Gm.+ rods.
4	Glass lined tank	190° F. 30 min. 3 hrs. at 100° F. 190° F. 30 min.	wheyed off, bad odor and flavor, bitter, large Gm.+ rods.
5	Glass lined tank	190° F. 30 min. 5 hrs. at 100° F. 190° F. 30 min.	satisfactory flavor and odor.
6	Glass lined tank	190° F. 1 hour. 3 hrs. at 100° F. 190° F. 1 hour.	fair flavor, good curd, few large Gm.+ rods, satisfactory.
7	Glass lined tank	185 - 190° F. 1 hour. 6 hrs. at 100° F. 185 - 190° F. 1 hour.	fair flavor and odor, firm curd, few large Gm.+ rods.

treatment varied from a single heating at 190 - 194° F. for 50 minutes to double exposures at 185 - 195° F. for 50 minutes.

In three trials fairly satisfactory products were obtained from milk treated in the glass lined tank. In trial five the same pasteurization exposure was used as in the two previous trials but the incubation period was five hours instead of three. On the last two trials the time of exposure at 185 - 190° F. was increased to one hour with different incubation periods in the two trials.

Further work was prevented because of the unfavorable effect which the heavy contamination of the tank was having on the ordinarily successful sterilized milk product. The results obtained seem to indicate, however, that with a high quality milk two exposures each of at least 190° F. for one hour with an incubation period of from three to five hours between will produce a commercially satisfactory product without the disadvantages of the sterilized milk. The higher quality of the product and lower number of contaminating organisms obtained by heating to 195 - 200° F., as in the trials with the milk in flasks, seem to make it advisable to use these exposures rather than the lower one of 190° F.

EFFECT OF DIFFERENT TEMPERATURES ON THE VIABILITY OF L. ACIDOPHILUS.

Practically all of the milk products whose condition is affected either favorably or unfavorably by microorganisms are stored at low temperatures. From the commercial standpoint the question of the palatability of a fermented milk is very important because of the natural aversion of most consumers to sour milk, while from a therapeutic standpoint the number of viable organisms present at the time of consumption seems to be equally significant. A low storage temperature appears to be most favorable in maintaining palatability while it has been pointed out by one investigator that the number of viable organisms decreases rapidly at refrigerator temperature but that for several days considerable numbers remain viable at room temperature. A number of trials were made with milk cultures held at various temperatures to obtain information on the viability of the organisms; it was thought that such information might be of considerable importance in determining the most suitable temperature for storage.

Methods.

In the study of the effect of holding temperatures on the viability of L.acidophilus the following procedure was employed. A liter of skimmed milk was

sterilized in a two-liter flask at 15# pressure for 22 minutes, cooled and inoculated with approximately 10 cc. of a 48 hour milk culture of the strain under observation. The flasks were incubated at 37° C. until the typical soft L.acidophilus curd formed, which usually occurred in from 36 to 48 hours. The curd was thoroughly broken up by shaking until it was of a smooth, creamy, consistency, and then plated on whey agar by the usual dilution method. Bits of sterile cotton were used to wipe the end of the pipette with which the milk was measured into the first water blank so that the pipette might be rinsed out and uniformity obtained throughout the series of experiments. Observing the proper aseptic precautions, the milk was next divided into the desired number of sterile liter Erlenmeyer flasks. These flasks were incubated at room temperature (approximately 25° C.), refrigerator temperature (5-8° C.), ice water temperature (slightly above 0° C.), and at 37° C. At the intervals indicated in the tables the milk was plated in duplicate or triplicate; the plates were incubated at 37° C. and counted after 4 to 5 days. It was realized that according to the recent work of Kulp (1926) all of the viable organisms might not grow except in an atmosphere of CO_2 but inasmuch as the results were all obtained under the

same conditions they were satisfactory for comparative purposes.

In order to study the effect of a temperature even lower than would be used in the commercial handling of acidophilus milk, cultures were frozen in the form of lacto, a dairy product developed at the Iowa Agricultural Experiment Station. It was also felt that if sufficient numbers of the organisms remained viable for a few days this product would offer a satisfactory source of L.acidophilus to those who disliked or could not take acidophilus milk.

The lacto mix was prepared with the materials in the same proportion as originally reported (Mortensen and Gordon - 1911) except that one ounce of a very high grade gelatin was substituted for the eggs in several instances. In the earlier runs the mixing and freezing was done in a five gallon tub freezer, the can and all utensils being thoroughly steamed over a jet before using. In later runs the gelatin was omitted, as the main interest was not in the body of the product, and smaller quantities were frozen in a one gallon hand freezer sterilized in the autoclave at 15# pressure for 30 minutes together with all spoons or ladles used in the preparation.

Immediately before and after freezing, samples were

removed for plating. The remainder was packed in paper containers and held in a mechanically refrigerated cabinet at approximately 3° F. At the intervals indicated in the tables samples were removed aseptically and placed in sterile Petri dishes. By warming very gently and tipping the dish back and forth slowly the lacto was melted and mixed; samples free from air were secured for plating. The plating, incubating and counting were conducted as described above in dealing with the plain acidophilus milk.

Results Obtained.

Table XV presents the data on the first of a series of trials carried out to determine the effect of the holding temperature upon the viability of L.acidophilus. The milk culture of A1 was plated at three hour intervals during the first day and twice thereafter at 24 hour intervals. The results indicate that a gradual increase takes place during the first day of holding and that the numbers at the end of the second day are lower in the case of the samples held at room temperature and at 37° C. than with the sample held in the refrigerator. In all three instances there was an increase in numbers, the final count being 102% of the original at 37° C., 127% at room temperature and 157%

T A B L E XV.

The effect of temperature on the viability of Lactobacillus acidophilus - culture A1.

Time in days	Room temperature: cols. per plate	Room temperature: cols. per cc. in millions	Refrigerator temperature: cols. per plate	Refrigerator temperature: cols. per cc. in millions	Incubator temperature: cols. per plate	Incubator temperature: cols. per cc. in millions
	74 71	725		725		725
1/4	103 96	995	84 59	715	105 74	895
1/2	126 104	1,150	106 83	945	103 82	925
3/4	79 -	790	79 76	775	119 114	1,165
1	116 114	1,150	134 121	1,275	112 95	1,035
2	109 76	925	118 110	1,140	76 73	745

at refrigerator temperature.

In the second trial a fourth sample was included and held in ice water in the refrigerator; the data are presented in Table XVI. The milk was plated twice the first day and three times at 24 hour intervals, and the refrigerator sample again in seven days. The same general results shown in the first trial were apparent in this; namely, a gradual increase in numbers during the first day or two followed by a decrease. At the end of the third day 104% of the original number were present at room temperature, 110% at refrigerator temperature, 91.5% in ice water, and 87% at 37° C. The sample held 11 days in the refrigerator showed 122% of the original number.

Three trials were made with culture A5 at three temperatures as shown in Tables XVII, XVIII, XIX. Since 37° C. was unquestionably not a suitable holding temperature from the commercial standpoint, no sample was held at that temperature. In these three trials the numbers of viable organisms apparently decreased in a more or less gradual and regular manner. In two of the three trials the larger counts after seven days were obtained with the two samples at refrigerator and ice water temperatures, while in the third the count was higher with the sample at room temperature. The percent of the original count

TABLE XVI

The effect of temperature on the longevity of Lactobacillus acidophilus; culture A1.

Time in days	Room temperature: cols. per plate	Room temperature: cols. per cc. in millions	Refrigerator temperature: cols. per plate	Refrigerator temperature: cols. per cc. in millions	Ice water temperature: cols. per plate	Ice water temperature: cols. per cc. in millions	Incubator temperature: cols. per plate	Incubator temperature: cols. per cc. in millions
							108	
							95	
							88	970
1/2	124		129		106		102	
	105		129		96		100	
	96	1,087	104	1,207	96	993	85	957
1	148		113		109		119	
	140		103		98		108	
	114	1,340	93	1,030	89	987	108	1,117
2	128		104		115		97	
	117		103		92		89	
	92	1,123	87	980	90	990	85	903
3	118		112		98		96	
	93		106		90		80	
	92	1,010	101	1,063	78	887	77	843
11			134					
			115					
			105	1,180				

TABLE XVII

The effect of temperature on the longevity of Lactobacillus acidophilus. Culture A5.

Time in days	Room temperature. cols. per plate	Room temperature. cols. per cc. in millions	Refrigerator temperature. cols. per plate	Refrigerator temperature. cols. per cc. in millions	Ice water temperature. cols. per plate	Ice water temperature. cols. per cc. in millions
	31 25	280				
1	266 234	250	249 233	241	251 221	236
2	219 210	215	240 218	229	244 -	244
3	217 205	211	244 228	236	235 232	234
4	237 201	219	261 237	249	214 206	210
5	172 156	165	189 170	180	152 131	142
6	142 140	141	256 254	255	151 142	147
7	130 123	127	217 211	214	142 117	130
14	37 33	35	130 125	128		
21	65 55	6	33 27	30		

TABLE XVIII

The effect of temperature on the longevity of Lactobacillus acidophilus. Culture A5.

Time in days	Room temperature: cols. per plate	Room temperature: cols. per cc. in millions	Refrigerator temperature: cols. per plate	Refrigerator temperature: cols. per cc. in millions	Ice water temperature: cols. per plate	Ice water temperature: cols. per cc. in millions
0	258					
	269					
	-	264				
1	217		240		195	
	218		246		216	
	227	221	248	245	-	206
2	224		212		170	
	224		233		219	
	-	224	-	223	224	204
3	163		200		104	
	183		206		155	
	-	173	222	209	198	152
4	124		134		157	
	147		145		162	
	157	143	191	157	178	166
5	105		121		111	
	114		150		140	
	114	111	175	149	140	130
6	72		132		117	
	82		159		149	
	83	79	171	154	173	146
7	38		96		106	
	54		112		113	
	43	45	128	112	154	124
14	18		22			
	25		25			
	25	23	32	26		

TABLE XIX.

The effect of temperature on the longevity of Lactobacillus acidophilus. Culture A5.

Time in days	Room temperature: cols. per plate	Room temperature: cols. per cc. in millions	Refrigerator temperature: cols. per plate	Refrigerator temperature: cols. per cc. in millions	Ice water temperature: cols. per plate	Ice water temperature: cols. per cc. in millions
0	492					
	538					
	586*	539				
1	206		254		286	
	207		301		336	
	219	211	367	307	-	311
2	200		192		493	
	234		194		494	
	289	241	200	195	505*	497
3	276		479		447	
	289		480		466	
	296	287	495*	485	484*	466
4	179		150		122	
	185		168		137	
	-	187	175	164	198	152
5	165		100		152	
	171		85		154	
	185	174	-	93	171	159
6	177		36		124	
	192		37		137	
	-	184	40	38	-	131
7	155				118	
	172				126	
	188	172			140	128

* Estimated.

obtained after seven days varied from 7% (6 days) to 76.5%, both with a refrigerator sample. The average of the percentages in the three trials were 41.9% at refrigerator temperature, 39% in ice water, and 31.3% at room temperature. In two of the trials the refrigerator and room temperature samples were held two weeks and in one of these three weeks. In one instance there was no significant difference while in the other the sample held at refrigerator temperature still showed 45.7% of the original count and the room temperature sample 12.5%. At the end of the third week of holding the same samples respectively showed 10.7% and 2% of the original numbers.

Although not indicated in the tables, the total acidities of the samples were determined in most cases and they were also judged as to their palatability. As might be expected the acidity of the samples held in the refrigerator and in ice water showed practically no change and the milk was still palatable even after the longer holding periods, whereas the samples held at room temperature were in all cases unpalatable due to the high acid, which in all instances increased to over 1% and reached as high as 1.9% from initial acidities of approximately 0.8%.

It is also of interest in this connection to note observations on two cultures which had been left in the cooler for eight and nine months respectively in the original forms in which they were received - one in milk and the other in a mineral oil jelly. The milk culture was plated on whey agar; the count obtained was 400,000 colonies per cc. Unfortunately the numbers originally present were not determined. A fairly large inoculation of the mineral oil jelly preparation into litmus milk showed that the organisms were still able to cause coagulation on the third day of incubation at 37° C. This agreed very well with the rate of growth of these organisms when first received.

The results obtained in ten trials on the effect of freezing on the viability of L.acidophilus are presented in Tables XX and XXI.

The results of the first four trials are presented in Table XX. It was first intended to hold the lacto only two or three days as it was felt that this represented approximately the length of time it would be held commercially but the counts held up so well and the body remained so satisfactory that the holding period was extended to seven days. In the first trial 39.8% of the original number survived after two days holding. In the

TABLE XX

Effect of freezing on longevity of *Lactobacillus acidophilus*. Culture A1.

Age	Trial-1		Trial-2		Trial-3		Trial-4	
	cols. per plate	cols. per cc. in millions	cols. per plate	cols. per cc. in millions	cols. per plate	cols. per cc. in millions	cols. per plate	cols. per cc. in millions
Fresh mix	54 69	615	71 78 90	797	84 92	880	120 129	1,245
Fresh frozen	67 68	675	61 71 89	736	57 68	625	74 90	820
1 day	42 47	445	48 49 65	540	30 36	330	252 283	268
2 days	23 26	245			210 256	233	150 206	178
3 days			49 50 56	516	55 78	67	148 276	21
6 days							81 96	9
7 days							36 47	4

TABLE XXI

The effect of freezing on the viability of *Lactobacillus acidophilus*. Culture A1.

Age	Cols. per plate	Cols. per cc. in millions	Time to coagulate milk	Age	Cols. per plate	Cols. per cc. in millions	Time to coagulate milk
	Trial - 5				Trial - 6		
Mix.	75 90	825		Mix.	72 72	720	
Fresh frozen	63 65	640	36 hours	Fresh frozen	62 63	625	24 hours
1 day	61 65	630	" "	1 day	50 61	555	36 hours
2 days	52 67	595	" "	2 days	42 47	445	" "
3 days	61 65	630	" "	3 days			
4 days	48 59	535	" "	4 days	31 35	330	" "
5 days	53 54	535	" "	5 days			
6 days	48 56	520	" "	6 days	31 40	355	" "
7 days	50 52	510	" "	7 days	35 38	365	" "

5 days	53 54	535	"	"	5 days			
6 days	48 56	520	"	"	6 days	31 40	355	" "
7 days	50 52	510	"	"	7 days	35 38	365	" "
	Trial - 7					Trial - 8		
Mix	46 49	475			Mix	94 100	970	
Fresh frozen	32 37	345	36 hours		Fresh frozen	61 68	645	36 hours
1 day	31 35	330	"	"	1 day	61 63	570	" "
2 days	29 31	300	"	"	2 days	41 53	470	" "
3 days	26 38	320	"	"	3 days	38 62	500	" "
4 days	29 31	300	48 hours		4 days	35 37	360	" "
5 days	35 41	380	"	"	5 days	40 59	495	" "
6 days	32 35	335	"	"	6 days	49 53	510	" "
7 days	37 54	455	36 hours		7 days	40 51	455	" "

Continued on page 128a.

TABLE XXI (continued)

Age	Cols. per plate	Cols. per cc. in millions	Time to coagulate milk		Age	Cols. per plate	Cols. per cc. in millions	Time to coagulate milk	
	Trial - 9					Trial - 10			
Mix.	130 160	1,450			Mix.	76 96	860		
Fresh frozen	97 104	1,005	36 hours		Fresh frozen	77 79	780	36 hours	
1 day	68 85	765	"	"	1 day	47 51	490	"	"
2 days	68 76	720	"	"	2 days	57 70	635	"	"
3 days	78 79	785	"	"	3 days				
4 days	74 77	755	"	"	4 days	35 42	385	"	"
5 days	45 57	510	"	"	5 days				
6 days	74 75	745	"	"	6 days	35 40	375	"	"
7 days	69 74	715	"	"	7 days	28 35	315	"	"

next three trials the number surviving after 3 days varied from 1.7% to 64.7% after three days, while in trial four only 0.3% remained after seven days. This was the lowest percentage obtained in the 7 trials during which the samples were held 7 days.

In the last six trials, besides the plate counts, observations were made on the effect of inoculating 100 cc. of sterile milk with 1 cc. of the lacto and incubating at 37° C. The time to coagulate was recorded to the nearest 12 hours. In most cases it will be noticed that the time was 36 hours which was approximately the time required for a regular culture of A1 to coagulate under the same conditions. When the curd formed was of the typical L.acidophilus type with a slight amount of whey, the odor pleasant, and stained slides showed little or no contaminating forms, it was considered satisfactory evidence that the plate counts represented the organisms used in the lacto and not contaminating forms. Such an assumption was also justified since the findings of numerous investigators on ice cream indicate that the principal sources of bacteria are the milk and cream used in the mix; similar results were obtained in the original investigations on lacto.

The data on the last six trials are presented in

Table XXI. In general there seems to be a gradual and fairly regular decrease in numbers; although the data show fluctuations from day to day these are felt to be within the range of experimental error when the nature of the material being plated is considered. The highest percent of the original number surviving after 7 days was 95.8% in trial 7, and the lowest was 36.6% in trial 10; the average for the six trials was 56.7%. If the low figure of 0.3% obtained in trial 4 is included in the average of all the seven day trials, the figure becomes 48.7%.

Discussion of Results.

A study was made of the preparation of acidophilus milk by a double pasteurization of the milk both in flasks and in commercially practical dairy equipment. The product prepared in flasks was of good quality when the milk was subjected to two periods of heating at not less than 195° F. for one hour with an incubation between of three hours at 37° C. Although the acidophilus milk would have been considered commercially satisfactory as far as its palatability was concerned, it was never free from at least a few large Gram-positive rods.

During seven trials in which milk was heated in either a 30 gallon Haughdahl starter can or a glass lined

steel tank it was found that a fairly satisfactory product could be prepared in bulk by double pasteurization. The starter can was not satisfactory in the two trials in which it was used; three of five lots prepared in the glass lined tank were of fair quality. Two exposures of one hour each at 190° F. with an incubation between of from three to six hours seemed to be necessary for favorable results.

From these results it appears that with a good quality milk a commercially suitable white acidophilus milk can be prepared by treating the milk by double pasteurization. The superiority of the fermented milk prepared in flasks over that prepared in bulk seems to justify the conclusion that two exposures at 195 - 200° F. for one hour with an incubation between of about three hours at 100° F. should be recommended for commercial purposes. Such milk can not be expected to have as good keeping qualities as a sterilized milk product because of the presence of contaminating organisms of a type which might cause deterioration. For the preparation of acidophilus milk for early consumption it is felt that this method is practicable if carefully controlled and a milk of high quality is always available.

In a study of the effect of temperature on the

viability of L.acidophilus it was found that in almost every instance a larger proportion of the original number of organisms in a milk culture were viable after seven days in the refrigerator or in ice water than when held at room temperature. This difference usually became apparent within two or three days.

The palatability of the milk held at the lower temperatures always remained good throughout the holding period while that of the milk held at room temperature fell off rapidly due to the increase in acidity. Further trials in which acidophilus milk was frozen in the form of lacto showed that large numbers survived freezing for at least seven days; the average of the percent surviving in six trials was 56.7%. These results agree with those found by Mortensen and Gordon (1911) in a study of the organisms surviving in lacto made by the usual method.

The results of these trials indicate that acidophilus milk kept at refrigerator temperatures will maintain its palatability much longer than at room temperature and that during the length of time that the milk would ordinarily be kept there is not an excessive decrease in the number of viable organisms. The results with the lacto seemed to indicate the possible value of this product as a means of supplying L.acidophilus to persons who would

otherwise not consume these organisms as the ordinary acidophilus milk.

GENERAL DISCUSSION OF RESULTS.

A study was made of 86 cultures of lactobacilli considered as belonging to three species - L.acidophilus, L.bulgaricus, and L.casei. The cultures were secured from various sources: 33 were from research and commercial laboratories and included 9 L.acidophilus, 7 L.bulgaricus, and 17 L.casei; 53 were isolated, 16 of which came from the fecal matter of humans and animals and were considered as L.acidophilus, while 37 came from raw milk and cheddar cheese and were considered as L.casei.

In the isolation of the L.acidophilus strains from fecal material the Heymann acetic acid bouillon method was found to be more certain than a dilution method or direct plating. Fewer failures resulted with the acid medium while other methods were apparently successful only under particularly favorable conditions. These results agree with those of various investigators.

The use of milk as an enrichment medium for the development of the lactobacilli when isolating these types from milk and cheese proved very satisfactory. The frequency with which L.casei cultures were secured from milk and cheese showed their prevalence in these products;

numerous investigators have reported similar results.

The size of the various organisms made possible a classification into two groups which was convenient for discussion; in one group the cells were classed as large, and in the other as small.

Several important characters were studied with the idea of determining whether or not a correlation existed which would be of value in identifying and classifying organisms belonging to these three species. The ability of the cultures to grow at various temperatures seemed to show some correlation between this character and the size of the organisms. For the most part the cultures classed as large among the L.bulgaricus and L.acidophilus groups were able to grow at 45° C., while those classed as small in these groups were unable to grow or grew very poorly at this high temperature. On the other hand the cultures classed as small were for the most part able to grow at room temperature and at 15-20° C., while those classed as large were for the most part unable to grow or grew poorly at these temperatures. Twelve cultures classed as large in the L.casei group failed to grow at any of these temperatures, while at 37° C. these cultures grew very slowly. Eleven L.casei cultures grew at 45° C., but rather poorly, while practically all except the 12

noted grew well at room temperature and at 15-20° C. All except one of the 11 were classed as small. It appears from these results that in general the large organisms grew better than the small at 45° C.; while the small organisms grew better than the large at the lower temperatures. Since L.bulgaricus has long been recognized as having a high optimum growth temperature the relationships just indicated seemed to be significant. Studies of other characters emphasized this relationship.

Thirteen trials were made using several different media and depressants to determine the effect of lowered surface tension of the medium on the growth of the organisms being studied. Preliminary trials and the first few reported in this study showed the superiority of sodium ricinoleate as a depressant over sodium taurocholate, sodium glycocholate, or sodium oleate so in most of the trials sodium ricinoleate was used. When the surface tension of the medium was depressed much under 40 dynes the first cultures failing to grow were apparently in the group classed as large. In medium X all of the 60 cultures tested grew at 40 dynes; at 37.4 dynes 23 of 82 cultures failed to grow - 16 of these were large and 7 small. In whey peptone broth at 39.0 dynes 9 of 87 cultures failed to grow and all were classed as large;

at 37.3 dynes 58 of 87 failed to grow and it was noticeable that the small L.acidophilus and L.bulgaricus strains were among the few which grew. In beef infusion bouillon 17 of 60 cultures failed to grow and 16 of these were classed as large. Among 72 cultures tried in medium M, 4 large strains of L.bulgaricus and L.acidophilus failed to grow without depressant; at 39.0 dynes 9 failed to grow and all were classed as large. From these results it is evident that a close relationship exists between the large L.acidophilus and L.bulgaricus organisms in respect to their ability to grow in media with reduced surface tensions. The results also seem to indicate that 40 dynes is a critical surface tension for the L.bulgaricus types, and that the small L.acidophilus and L.casei types are able to grow when the surface tension is reduced to nearly 37 dynes. The suitability of the medium without depressant as well as the depressant used undoubtedly influence the ability of the organisms to grow at reduced surface tensions.

A study of the type of lactic acid produced in milk by 12 cultures considered as L.acidophilus showed variations from pure active to practically pure inactive acid. The active acid was always the dextro modification. Four of six large cultures produced largely or entirely inactive acid. In other characters, such as growth at

45° C., but not at 15-20° C., failure to grow in medium X at 37.4 dynes, and the failure of one of them to ferment maltose, these cultures closely resembled the L.bulgaricus cultures classed as large. These four were among five from other laboratories studied for the type of lactic acid produced.

Determinations of the total and volatile acids produced in milk by 66 cultures did not show any particularly striking relationships. Five of the six cultures producing over 2.0% acid were classed as large, grew at 45° C. but not at 15-20° C., and failed to grow at the lower surface tensions; two of these were L.acidophilus from laboratory sources, and three were L.bulgaricus. The volatile acidities varied widely, the values ranging from 4.7 to 41.7. The L.acidophilus cultures seemed to produce slightly higher volatile acidities since the values obtained with several were over 30, while none over 30 were obtained with the L.bulgaricus cultures and with only an occasional L.casei culture.

Sixteen cultures were studied as to their proteolytic activity in milk both with and without $CaCO_3$ during an incubation of 30 days. All of the cultures caused some increase in soluble nitrogen; the values varied from 1.0 to 6.7% in the milk without $CaCO_3$, and from

1.3 to 19.2% in the milk with $CaCO_3$. In nearly all instances a culture caused higher increases in milk with $CaCO_3$ than in milk without the carbonate. Among the groups of organisms when a culture caused the highest increase in milk with $CaCO_3$ it also caused the highest increase in milk without $CaCO_3$. No particularly significant relationships seemed to exist between the amount of proteolysis and other characters.

Studies of the various characters of the organisms seemed to bring out correlations which would justify certain conclusions as to their interrelationships. Most of the cultures classed as large in the L.acidophilus groups exhibited characteristics which indicated close relationship to the L.bulgaricus cultures classed as large. Among these characters were a tendency to grow better at 45° C. than at room temperature or 15 - 20° C.; a failure for the most part to grow at surface tensions much under 40 dynes; a tendency to fail to ferment maltose, particularly under a reduced surface tension; and a production of approximately the same amounts of lactic acid, which was also mainly the inactive modification. The small L.acidophilus types on the other hand showed close relationship to the L.casei cultures in these characters; their reactions to the conditions mentioned above have

been pointed out as being opposite to those shown by the large types. It seems that many of the large types previously considered as L.acidophilus should have been considered as L.bulgaricus and the small types as L.casei or else that two types of L.acidophilus exist one of which is closely related to L.bulgaricus and the other to L.casei. If the latter view is to be accepted, L.casei seems to bear closer relationships to both L.acidophilus and L.bulgaricus than these two species do to one another. If any one species is to be considered a central type, it appears from this study that L.casei rather than L.acidophilus should be accepted as the central type. In any case it must be recognized that certain forms are found which apparently are borderline strains lying between two species so that no absolutely definite line of demarcation can be drawn between them. The close relationship between the L.casei and L.acidophilus types suggested the probability that L.casei could be implanted in the intestinal tract since indications are that L.acidophilus can be.

Feeding experiments were conducted with four young men and with rats; the effects of feeding were determined by bacteriological studies of the feces. The men consumed daily, with the regular diet, one quart of milk

fermented with several L.casei cultures isolated from milk and cheese. The rats were fed suspensions of pure cultures of single strains of L.casei or L.acidophilus with a basal diet of fresh white bread, and fresh beef; lactose was added to the diet of all except the control rats during the latter part of the trials. Results obtained with the men particularly seemed to justify the conclusion that transformation of the intestinal flora took place. Less definite results were obtained with the rats although the change in flora was sufficient for an unbiased observer to be able to detect under the microscope which fecal smears were from rats receiving cultures and which were from the controls. No such proportions of Gram-positive organisms were found in any fecal smears as have been reported by several investigators. The results were more in agreement with those reported by Kopeloff (1926).

The ability to become implanted in the intestinal tract further emphasized the close relationship between L.casei and L.acidophilus, particularly between the small strains. It also indicated the probability that many reports of favorable changes in the intestinal flora from milk feeding have been due in part at least to the presence of L.casei types in the milk. The importance to the dairy

industry of the prevalence of these types in milk and cheese is further brought out by the results of these trials.

It was found to be possible to produce a satisfactory white acidophilus milk by two exposures of a good quality milk at 195 - 200° F. for one hour each, with an incubation between of about three hours at 100° F. Large Gram-positive rods, very evidently not L.acidophilus, were present, however, in every instance. This method was not as consistently successful as when the milk was sterilized.

Storage of acidophilus milk at refrigerator temperature was found to be more satisfactory than at room temperature both from the standpoint of palatability and the number of organisms remaining viable after seven days. The organisms were also found to be able to survive freezing in the form of lacto; the average of six trials showed that 56.7% of the original number were viable after seven days. This was considered a new and satisfactory medium for supplying viable L.acidophilus to persons who would not take acidophilus milk in the usual form.

CONCLUSIONS.

1. The Heymann acetic acid bouillon method of isolating L.acidophilus from fecal matter proved more certain than a dilution method or direct plating.

2. The use of milk as an enrichment medium was very satisfactory for the isolation of L.casei from milk and cheddar cheese.

3. The size of the lactobacilli studied showed certain correlation with the temperatures at which they were able to grow. In general the large types grew better than the small at 45° C., while the small grew better than the large at room temperature and 15 - 20° C.

4. The size of the lactobacilli studied showed certain correlation with their ability to grow in media with reduced surface tensions. In general the small types were able to grow at lower surface tensions than the large.

5. The critical surface tension for L.bulgaricus in the media used was apparently 40 dynes; L.acidophilus and L.casei were able to grow when the surface tension was at least 2 to 3 dynes lower.

6. Twelve L.acidophilus cultures produced largely dextro lactic acid.

7. The amount of total and volatile acids produced by 66 cultures of the three species varied widely. All produced some volatile acid.

8. The soluble nitrogen in milk with and without $CaCO_3$ was increased by 16 cultures including representative members of the three species.

9. The L.casei cultures were very closely related to both L.acidophilus and L.bulgaricus. Either two types of L.acidophilus exist or else the large types should be classed as L.bulgaricus and the small types as L.casei. If the two type idea is accepted, L.casei rather than L.acidophilus should be considered as the central type of these species.

10. L. casei seemed to be capable of implantation in the intestinal tract of humans and rats.

11. It was found to be possible to prepare white acidophilus milk by pasteurization of the milk at 195 - 200° F. with two exposures of one hour each and an incubation period of about 3 hours at 100° F. between. Not as consistently good results were obtained as by sterilization of the milk, and large Gram-positive rods, very evidently not L.acidophilus, were always present.

12. Refrigerator temperature was found to be better than room temperature for the storage of acidophilus milk; the milk remained palatable longer, and more organisms were viable after 7 days at refrigerator temperature.

13. A large proportion of the viable organisms survived freezing in the form of lacto for 7 days.

14. Acidophilus lacto was considered a new and satisfactory medium for supplying viable L.acidophilus to consumers.

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